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DISRUPÇÃO ENDÓCRINA NO ISÓPODE TERRESTRE *PORCELLIO SCABER*

ENDOCRINE DISRUPTION IN THE TERRESTRIAL ISOPOD *PORCELLIO SCABER*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Amadeu Mortágua Velho da Maia Soares, Professor Catedrático do Departamento de Biologia da Universidade de Aveiro e do Doutor Cornelis Adrianus Maria Van Gestel, Professor Associado do Departamento de Ecologia Animal da Universidade Livre de Amesterdão.

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palavras-chave

20-hidroxiecdisona • Bisfenol A • Disrupção endócrina • Efeitos de baixas concentrações • Isópodes terrestres • *Porcellio scaber* • Proteómica • Toxicidade na reprodução • Toxicidade no desenvolvimento • Vinclozolina

resumo

Nas últimas décadas tem-se assistido a uma preocupação crescente relativamente às possíveis consequências da exposição a compostos xenóbioticos capazes de modular ou causar disrupção do sistema endócrino, os denominados Compostos Disruptores Endócrinos (CDEs). A maioria dos estudos efectuados tem-se centrado principalmente nos efeitos dos CDEs em vertebrados, enquanto que os seus efeitos em invertebrados têm sido negligenciados, embora este grupo represente mais de 95% de todas as espécies animais.

Isópodes como o *Porcellio scaber*, combinam características associadas às mudas e aos processos reprodutivos mediados por mecanismos endócrinos conhecidos com um modo de vida terrestre, tornando-os potenciais espécies sentinela para estudos de disrupção endócrina (DE) em ambientes terrestres.

Neste estudo, isópodes machos adultos, machos e fêmeas juvenis e casais foram expostos a concentrações crescentes de dois CDEs, vinclozolina (Vz) e bisfenol A (BPA). Testou-se a hipótese nula que a Vz e o BPA não interferem com o desenvolvimento e reprodução deste isópode terrestre. Foi investigada a possível ligação entre os efeitos causados pelos compostos propostos e DE assim como a ligação a outros potenciais mecanismos de toxicidade. Parâmetros como concentração de 20-hidroxiecdisona (20E), muda, crescimento, rácios sexuais e diversos parâmetros reprodutivos foram estudados. Adicionalmente, de modo a estudar os alvos moleculares destes tóxicos, analisou-se a expressão proteica do intestino, hepatopâncreas e testículos do isópode após exposição aos químicos.

Os resultados demonstram que a Vz e o BPA estimulam o aumento dos níveis de 20E de um modo dependente da dose. Excepção feita para a concentração mais baixa de BPA testada (10 mg/kg solo), para a qual concentrações significativamente mais altas de 20E foram determinadas, sugerindo a ocorrência dos “efeitos de baixas doses típicos de DE” já demonstrados por outros autores. O BPA também distorceu o rácio sexual favorecendo as fêmeas na concentração mais baixa. A mortalidade devido à ecdise incompleta foi relacionada com o hiper-ecdisonismo nas concentrações mais elevadas de Vz. Mais ainda, a Vz tende a atrasar a muda e o BPA a induzi-la. Não obstante, ambos os compostos provocam toxicidade no desenvolvimento, uma vez que foi encontrada uma diminuição generalizada nos parâmetros de crescimento. Os juvenis mostraram ser mais sensíveis à exposição aos tóxicos que os adultos. Estes compostos provocaram ainda toxicidade reprodutiva, com um decréscimo generalizado do “output” reprodutivo. A toxicidade causada pelos ecdisteróides e o seu papel na síntese de vitelogenina são alguns dos factores chave que poderão influenciar negativamente a reprodução. A Vz e o BPA afectaram a expressão de proteínas envolvidas no metabolismo energético e induziram várias respostas de *stress*. Interferiram ainda com proteínas intimamente ligadas com o sucesso reprodutivo.

Conclui-se assim, que ambos os CDEs propostos provocam toxicidade no desenvolvimento e na reprodução de *P. scaber*, tendo sido evidenciada uma ligação a DE. Alvos moleculares de natureza não-endócrina foram também revelados, através da expressão diferencial de algumas proteínas previamente descritas para invertebrados aquáticos e mesmo alguns vertebrados.

keywords

20-hydroxyecdysone • Bisphenol A • Developmental toxicity • Endocrine disruption • Low-dose effects • *Porcellio scaber* • Proteomics • Reproductive toxicity • Terrestrial isopods • Vinclozolin.

abstract

In the past few decades there has been a growing concern about the possible consequences of exposure to xenobiotic compounds that are able to modulate or cause a disruption in the endocrine system, the Endocrine Disrupting Compounds (EDCs). The majority of research done has focused mainly on the effects of EDCs on vertebrates while the effects on invertebrates have been largely ignored, despite this group representing more than 95% of all animal species.

Isopods like *Porcellio scaber* combine the features of molting and reproductive processes mediated by known endocrine mechanisms with a terrestrial mode of life, making them potential sentinel species for the study of endocrine disruption (ED) in soil environments.

In this study, male adult isopods, male and female juveniles, and couples were exposed to increasing soil concentrations of two proposed EDCs, vinclozolin (Vz) and bisphenol A (BPA). The null hypothesis was that Vz and BPA does not disrupt the normal development nor compromise the reproduction of the terrestrial isopod *P. scaber*. A causal link to ED and to other potential mechanisms by which these chemicals could elicit toxicity was addressed.

Several parameters such as 20-hydroxyecdysone (20E) levels, molting parameters, growth, sex ratio and several reproductive parameters were assessed. Furthermore, to study a vast array of molecular targets, the protein expression of the isopod gut, hepatopancreas and testes was investigated.

Results demonstrated that both Vz and BPA induced increased 20E levels in a dose-dependent way. An exception was BPA at the lowest concentration tested (10 mg/kg soil), at which high levels of 20E were also found, suggesting the presence of "ED typical low-dose effects". BPA also induced a sex ratio shift favouring females at the lowest concentration. Mortality due to incomplete ecdysis was related to hyperecdysonism at the highest concentrations of Vz. Also, Vz tended to postpone molt and BPA induced it. Nevertheless, both compounds elicited developmental toxicity since an overall growth decrease was found. Juveniles were more sensitive to toxicant exposure than adults. These compounds also elicited reproductive toxicity with an overall decrease of the reproductive output. Ecdysteroidal toxicity and its role in vitellogenin synthesis are some of the key factors possibly causing the reproductive impairment. BPA and Vz affected proteins involved in energy metabolism, induced a variety of stress responses and interfered with proteins intimately linked with the isopods' reproductive success.

It is concluded from this investigation that Vz and BPA elicit reproductive and developmental toxicity and a causal link to ED was provided. Non-endocrine molecular targets were also revealed for both compounds, with a differential expression of some proteins previously reported for some aquatic invertebrates and also some vertebrates.

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ABBREVIATIONS

20E	20-hydroxyecdysone
2-DE	two-dimensional gel electrophoresis
A.i.	active ingredient
ACN	acetonitrile
ACR	acute:chronic ratio
ACTH	adrenocorticotropic hormone
AK	arginine kinase
ANOVA	analysis of variance
ASTM	American Society for Testing and Materials
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BICINE	n,n-bis(2-hydroxyethyl) glycine
BPA	bisphenol A; 4,4'-dihydroxy-2,2-diphenylpropane
CAS	Chemical Abstracts Service
CI	confidence intervals
CW	cephalothorax width
DF	degrees of freedom
DIEA	n,n-diisopropylethylamine
DIGE	differential in gel electrophoresis
DNA	deoxyribonucleic acid
DW	dry weight
ECD	endocrine disruptor compound
EC	European Commission
EcR	ecdysone receptor
ED	endocrine disruption
EIA	enzyme immunoassay
ER	endoplasmic reticulum
EU	European Union
GC-MS	gas chromatography-mass spectrometry

GRP	glucose-regulated protein
GuHCl	guanidine hydrochloride
HPLC-PDA	high-performance liquid chromatography - photo diode array
Hsp	heat shock protein
IGR	insect growth regulator
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
LC	lethal concentration
LOEC	lowest observed effect concentration
MALDI	matrix-assisted laser desorption ionization
MIH	molting inhibiting hormone
MF	methyl farnesoate
MO	mandibular organ
MoA	mode of action
MQ	ultra-pure water
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NOEC	no observed effect concentration
NP	4-nonylphenol
OD	optical density
OECD	Organisation for Economic Co-operation and Development
PAH	polycyclic aromatic hydrocarbons
PET	polyethylene terephtalate
PMSF	phenylmethylsulphonyl fluoride
RA	reproductive allocation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	standard error
TBT	tributyltin
TFA	trifluoroacetic acid
TOF	time of flight

TRIS	2-amino-2-hydroxymethylpropane-1,3-diol
US	United States of America
US EPA	United States Environmental Protection Agency
vtg	vitellogenin
Vz	vinclozolin; 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione

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Chapter 1

General Introduction

1. General Introduction

1.1 Endocrine Disruption

In the past few decades concern has been growing about the possible consequences of environmental exposure to a group of chemicals (natural, synthetic, industrial chemicals or by-products), which are suspected to alter the functions of the endocrine system and consequently of causing adverse health effects in an intact organism, its offspring, or (sub) population (European Commission, 2007), the Endocrine Disruptor Compounds (EDCs). Today, this concern is focused both towards human health and to the impacts on wildlife and the environment, being already a priority in research and legislation within the European Union (European Commission, 1999, 2001, 2004, 2007), the US Environmental Protection Agency (Kavlock et al., 1996; U.S. EPA, 1998; Harding et al., 2006) and the World Health Organization (Damstra et al., 2002).
All known vertebrate and invertebrate taxa use chemical signalling molecules (hormones). Changes of the endocrine function can be felt when EDCs interfere with the synthesis, secretion, transport, action or elimination of natural hormones, which are responsible for homeostasis mechanisms, reproduction, growth and/or behaviour. These interferences can be caused by the direct binding of EDCs to receptors – acting as hormone mimics (agonists) or as "anti-hormones" (antagonists) – or indirectly by modulating endogenous hormone levels by interfering with biochemical processes associated with the production, availability, or metabolism of hormones or also by the modulation of their receptors.
Although invertebrates dominate over 95% of the known animal species and represent more than 30 different *phyla* within the animal kingdom (Ruppert et al., 2003), potential effects of suspected EDCs on the various invertebrate endocrine systems have not been studied with comparable intensity as in vertebrates, especially in fish (e.g. Baker et al., 2009), reptiles (e.g. De Falco et al., 2007),

amphibians (e.g. Kaneko et al., 2008), birds (e.g. Halldin et al., 2001) and mammals (e.g.Tabuchi et al., 2006).

Although the issue of Endocrine Disruption (ED) in invertebrates received an increasing scientific interest in the past, only a limited number of confirmed cases were reported (deFur et al., 1999). These are clearly dominated by insect growth regulators (IGRs), which were designed to act as EDCs for the use as insect pest control, and by studies on the antifouling biocide tributyltin (TBT) that was shown to induce imposex and intersex in prosobranch snails (Matthiessen and Gibbs, 1998). Imposex has been associated with skewed sex ratios, reduced fecundity, population declines, and local extinctions of affected gastropod populations (Gibbs and Bryan, 1986). These are perhaps the most complete examples of ED in wildlife populations. Further examples for ED in invertebrates are scarce and limited to laboratory studies, where compounds exhibited effects on endocrine regulated processes in marine and freshwater invertebrates (Porte et al., 2006). Endocrine changes following exposure to certain compounds may therefore be missed or simply be immeasurable at present, even though there is increasing evidence indicating that invertebrates are susceptible to ED (Porte et al., 2006).

Consequently, there is no reason to suppose that far-reaching changes as demonstrated by TBT and its effects on prosobranch populations are in any sense unique within the invertebrates (Matthiessen and Gibbs, 1998).

The “Endocrine Disruption in Invertebrates: Endocrinology, Testing, and Assessment” report (deFur et al., 1999) summarizes about 56 studies where ED may have occurred in invertebrates, although non-endocrine mechanisms are also possible for the observed effects. Effects like reduced molting frequency, reduced fecundity, elevated ecdysteroid levels, delayed reproduction, reduced size of neonates, increased brood size, mortality, increased intermolt duration, delayed maturation, impairment of reproduction, reduction in moult frequency, reduced egg production, delayed brood release, reduced elimination of testosterone metabolites, retardation of regenerative limb growth and molting, suppression of ovarian growth, differential sex ratio and super-female induction, have been summarized in this report (deFur et al., 1999). This includes several studies which comprise many compounds suspected of being hormonally active on aquatic crustaceans.

The crustaceans represent the group of invertebrates that provide the majority of ED case studies. Nevertheless, while the examples for the aquatic environment are almost balanced between freshwater and marine species, to our knowledge there are no reports of comparable effects in terrestrial crustaceans.

With the exception of TBT effects in molluscs, that have been associated with a locally severe impact on community levels (Matthiessen and Gibbs, 1998), and IGRs in target terrestrial insects, there are only a few field examples of ED in invertebrates. Nevertheless, much more examples for ED affecting invertebrate populations and communities can be expected, though still undetected. This assumption is supported by a number of indications, such as:

- chemical signalling systems and their basic mechanisms in the animal kingdom exhibit a considerable degree of conservatism (McLachlan, 2001). Consequently, endocrine systems in invertebrates can be presumed to be subject to modulation by identical or similar compounds as in vertebrates (Pinder and Pottinger, 1998);

- highly effective EDCs have been intentionally developed for the purpose of pest control to interfere with hormonal systems of insects. Such endocrine-mediating properties can be assumed as not being unique for the IGRs or this group of arthropods (Oehlmann and Schulte-Oehlmann, 2003).

Numerous studies provide strong evidence of effects on development, fecundity and reproductive output of invertebrates that can be attributed to substances acting as EDCs (Gibbs and Bryan, 1986; Matthiessen and Gibbs, 1998; Pinder and Pottinger, 1998; Oehlmann and Schulte-Oehlmann, 2003). So, carefully targeted monitoring programs are needed because effects in invertebrates are probably widespread but undetected (Fent, 2004).

1.2 Endocrine-disrupting chemicals

In June 2000 a list of 564 potential EDCs was published in a report of BKH Consulting Engineers, Delft, and TNO Nutrition and Food Research, Zeist, both from The Netherlands. This list of substances was completed having in mind the persistence in the environment, production volume, the scientific evidence of endocrine disruption and wildlife and human exposure. These criteria were used to categorise the candidate substances. From these, a group of 60 compounds considered to have endocrine disrupting activity (have shown endocrine activity in at least one in vivo study before) and for which a high level of concern existed with regard to exposure, deserved a special attention. These 60 compounds were included in a high priority list of EDCs proposed by the EU Commission (European Commission DG ENV, 2002). This high priority list includes agrochemicals or crop protection agents (e.g. lindane, vinclozolin, linuron, diuron, the common metabolite of linuron and diuron, 3,4-dichloroaniline, as well as triphenyltin compounds), biocides with antifouling properties (tributyltin compounds), and industrial chemicals including plasticizers (e.g. benzyl-n-butylphthalate, di-n-butylphthalate, bisphenol A) or flame retardants (e.g. PBBS).

In this study, two compounds from the EU highest priority list were selected: the fungicide vinclozolin (anti-androgen) and the industrial chemical bisphenol A (xeno-oestrogen).

Vinclozolin [Vz, 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione] is a non-systemic dicarboxymide fungicide, manufactured by BASF and commercially sold under the names Ronilan® (50% active ingredient), Curalan®, and Ornilan® (figure 1.1). It is efficient in controlling plants or fruit diseases caused by *Botrytis* spp., *Monilia* spp., or *Sclerotinia* spp. (Bursztyka et al., 2008) that affect crops such as lettuce, raspberries, beans and onions (Price et al., 2007). This fungicide is widely used in the United States of America and throughout Europe. In Britain, as well as in Germany, up to 50 tonnes of vinclozolin are used each year and it was estimated that in 2002, in the USA, 2,330,738 US dollars were spent on vinclozolin for crop protection (Gianessi and Reigner, 2005).

When sprayed as Ronilan®, at the maximum recommended application rate, the concentration of vinclozolin in the soil is 1 mg a.i./kg (assuming that 70 % of the fungicide will reach the surface and is homogeneously distributed over the top 5 cm soil layer and the soil bulk density is 1.4 kg/dm^3) (Lemos et al., 2009). Vinclozolin has a low to moderate persistence in soil, with reported half-lives from 28-43 days in the laboratory up to 34-94 days in the field and 6-12% of the original compound is present after 1 year (U.S. EPA, 1991; IUPAC, 2006). On plant leaves, vinclozolin is detectable on the leaf as the parent compound but does not wash off, since it is more soluble in oil than in water. This implies that vinclozolin does not wash off easily from foods (Szeto et al., 1989).

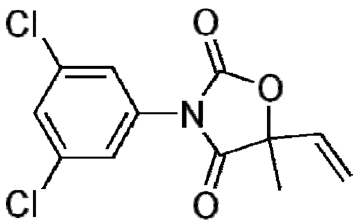

Figure 1.1 - Chemical structure of vinclozolin.

Vz anti-androgenic (‘anti-maleness’) effects have been deeply investigated in vertebrates and it is known that it inhibits testosterone induced growth of androgen-dependent tissues (Kang et al., 2004). The anti-androgenic effects caused by this substance are due to two (M1 and M2) of its 15 metabolites, which are able to bind to the androgen receptor (Gray, 1998; Vinggaard et al., 1999). These metabolites are responsible for the anti-androgenic effects attributed to Vz since they competitively inhibit the binding of androgens to the human androgen receptor and are 100 and 10-fold (respectively) more active than Vz (Kelce et al., 1997; Anway et al., 2005; Kavlock and Cummings, 2005). It has been reported that these metabolites may be produced both spontaneously in the presence of aqueous buffers and by biotransformation of Vz (Bursztyka et al., 2008).

Metabolites have also been found in human food (Gonzalez-Rodriguez et al., 2008).

Vz endocrine disruptor effects include induction of Leydig cell tumors, reduction of ejaculated sperm numbers and prostate weight, and delayed puberty in exposed rats. One major concern is that Vz causes transgenerational effects. F1 to F4 generations of male rats exposed to Vz at the time of gonadal sex determination developed prostate disease, kidney disease, immune system abnormalities, spermatogenesis abnormalities, breast tumour development, and blood abnormalities as hypercholesterolemia, which have been associated with an alteration in the epigenetic programming of the male germ line (Anway et al., 2006; Anway and Skinner, 2008). Similar effects have been shown for pregnant rat exposed to Vz, where a transgenerational increase in pregnancy abnormalities and female adult onset disease states are promoted (Nilsson et al., 2008).

The existing information supports the hypothesis that vinclozolin steroid-mediated actions in vertebrates have similar sub-lethal effects in invertebrates. In *Daphnia magna* it induces a decrease in the number of newborn males (Haeba et al., 2008). In molluscs Vz was shown to exert anti-androgenic effects, such as reduction of ejaculated sperm cells, smaller testes and disrupted male courtship behaviour (Baatrup and Junge, 2001), and reduced penis length and accessory male sex organs in prosobranch snails species (Tillmann et al., 2001). Vz has also been reported to cause female virilisation (imposex development) and reduction of accessory sex organ expression in the fresh water snail *Marisa cornuarietis* and two marine prosobranchs *Nucella lapillus* and *Nassarius reticulates* (Tillmann et al., 2001).

Bisphenol A [BPA, 2,2-bis-(4-hydroxyphenyl)-propane; figure 1.2] is a xenobiotic commonly employed in the manufacture of polycarbonate plastic and epoxy resins (Crain et al., 2007). It is released into the environment through sewage treatment effluent (Meesters and Schroder, 2002), landfill leachate (Wintgens et al., 2003), or natural degradation of polycarbonate plastics (Crain et al., 2007). Approximately 1.7 billion pounds of BPA are synthesized and used in the United States per year, classifying BPA as a high production volume chemical (Crain et al., 2007).

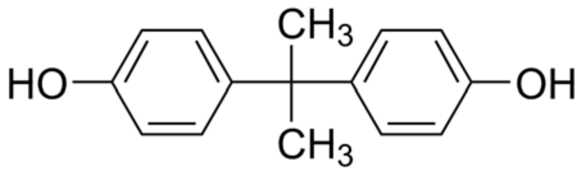

Figure 1.2 - Chemical structure of bisphenol A.

The only significant route of BPA to the terrestrial environment is through the application of sewage sludge from municipal plants [concentrations of 0.033-36.7 mg/kg (dw)] (Lee and Peart, 2000) as soil improvers. The half-life for bisphenol-A in soil was calculated as from 3 days (Fent et al., 2003) up to 37.5 days (based on modelled half-life in water) (Environment Canada, 2008). BPA has been shown to leach from water bottles and food cans into the packaged foodstuffs (Quitmeyer and Roberts, 2007). It then enters the body through the digestive tract when these foods are consumed. The level of BPA released from plastic depends on the age and wear of the plastic and on exposure to heat. BPA is also present in rivers and streams and in drinking water, presumably due to leaching from plastic items in landfills (Kuch and Ballschmiter, 2001; Kolpin et al., 2002; Coors et al., 2003). A survey by the Centre for Disease Control and Prevention found that approximately 95% of Americans have detectable levels of BPA in their bodies (Calafat et al., 2005). Therefore, in humans, dietary consumption is the most important exposure route (Biles et al., 1997; Wilson et al., 2007), since BPA's main sources of exposure are liquid and food storage containers. The sources of environmental contamination are either sewage treatment effluent (via human-ingested BPA being eliminated through sewage), landfill leachate (via hydrolysis of BPA from plastics), or natural degradation of polycarbonate plastics. Whereas sewage effluents and landfill leachates are point sources of BPA in the environment, fragments of epoxy resins and polycarbonate plastic debris entering the watershed through runoff are non-point sources, creating challenges for remediation (Crain et al., 2007).

Despite the claim from the plastic industry that BPA is safe, studies on animals have suggested that BPA has the potential to disrupt normal hormonal signalling by mimicking oestrogen, acting as an oestrogen receptor agonist (Krishnan et al., 1993). Also some anti-androgenic properties were identified (Sohoni and Sumpter, 1998), raising concerns about the potential of BPA to cause harm to humans (Quitmeyer and Roberts, 2007). Furthermore, recent findings suggest that BPA may be associated with increased risk of diabetes and cardiovascular diseases in humans (Lang et al., 2008).

BPA is known to act as a teratogen (although only at unrealistically high dosages) (Crain et al., 2007) and at realistic environmental doses, as an EDC in vertebrates (Safe et al., 2000). In vertebrate wildlife species, BPA induces alteration of sex determination during gonad organogenesis or alteration of gonad function during and after gonad organogenesis (Crain et al., 2007). In invertebrates, BPA also had adverse effects on gonad function inducing effects on reproduction of the water flea *Ceriodaphnia dubia* (Tatarazako et al., 2002), on female fecundity of *Daphnia magna* (Mu et al., 2005), on the structure and physiology of *Hydra vulgaris* polyps (Pascoe et al., 2002) and on the time to achieve sexual maturity of *Tigriopus japonicus* (Marcial et al., 2003). Nevertheless, at environmentally relevant doses there are only a few reports of reproductive effects among invertebrates, which include the superfeminization syndrome of BPA exposed organisms (Oehlmann et al., 2000; Duft et al., 2003; Jobling et al., 2004).

1.3 Endocrine system of invertebrates with special reference to crustacean endocrinology

As stated before, although 95% of known species in the animal kingdom are invertebrates (Ruppert et al., 2003), relatively little is known about their endocrine systems, making the studies on endocrine disruption rather difficult. Nevertheless, both vertebrates and invertebrates use hormones to regulate biological processes. These chemical mediators exert their regulatory effect at low concentrations, at various target sites within the body. They are responsible for the regulation of biological processes such as reproduction, development, behaviour, immunological, nervous, and homeostatic mechanisms.

In invertebrates, most often internal secretory structures are of neuronal origin, and are called “neurosecretory cells” or “organs”. These structures are the basis for the invertebrate hormones that comprise a variety of molecular structures, including steroids, terpenoids, and peptides. From these, the most important for crustaceans and insects are the non-peptide endocrine messengers such as ecdysteroids (Chang et al., 2001). In crustaceans, the biosynthesis of ecdysteroids occurs primarily in the Y-organ, the homologue of the insect prothoracic gland and are then rapidly hydroxylated in several tissues to 20-hydroxyecdysone (20E), the physiologically active form of the arthropod molting hormone (figure 1.3).

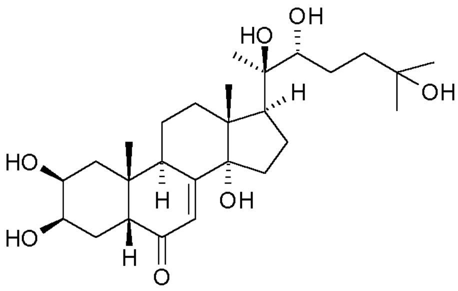

Figure 1.3 - Chemical structure of 20-hydroxyecdysone.

The Y-organ is under the inhibitory control of the molting inhibiting hormone (MIH), a neuropeptide secreted by the X-organ sinus gland, while methyl farnesoate (MF), a sesquiterpenoid compound secreted by the mandibular organ (MO), is involved in the stimulation of ecdysteroid synthesis by the Y-organ [see Subramoniam (2000) for a review] (figure 1.4).

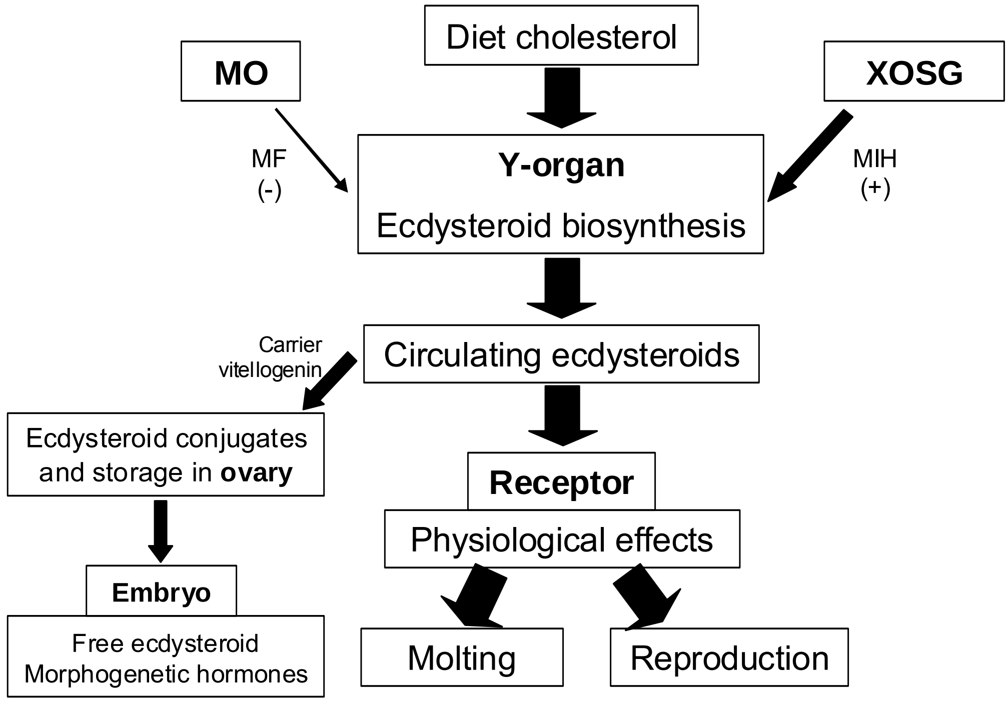

Figure 1.4 - Diagram of the control of ecdysteroid synthesis and catabolism and its main roles in crustacean physiology. XOSG, X-organ sinus gland complex; MO, mandibular organ; MF, methyl farnesoate; MIH, molt inhibiting hormone; +, stimulation; -, inhibition.
Adapted from Subramoniam (2000).

The molting of arthropods like insects and crustaceans requires periodic loss of the exoskeleton and replacement with and hardening of a new larger cuticle. This process is under the regulation of ecdysteroids (Horn et al., 1966). Hemolymph ecdysteroid levels during intermolt periods are low, but during the onset of the mid premolt stage levels of this hormone sharply rise. With the decline of 20E titres in the organism by stage D_2 of premolt, the organism starts the production of the new cuticle. When basal levels of the ecdysteroid are attained, a series of neuropeptides are released causing shedding of the old cuticle by a process termed ecdysis (Subramoniam, 2000) (figure 1.5).

Ecdysteroids are mostly known for their role in regulating molting but they also play a role in embryo development, diapause, cuticle formation, ovulation and spermatogenesis (Gunamalai et al., 2004). In terrestrial isopods, the female reproductive cycle (ovarian maturation and embryogenesis) is always a overlapping/synchronous event along with the molt cycle (Subramoniam, 2000). Ecdysteroids, as chief hormonal factors (Chang et al., 2001), are also necessary for vitellogenin synthesis (Vafopoulou and Steel, 1995). The coordinated control of molting and reproduction is achieved by this ecdysteroid/vitellogenin inter-relationship with each reproductive cycle having the length of one molt cycle, and with vitellogenic processes being completed during an extended intermolt period, with spawning occurring after ecdysis. Accordingly, any significant impact on the molting process might impair the reproductive success.

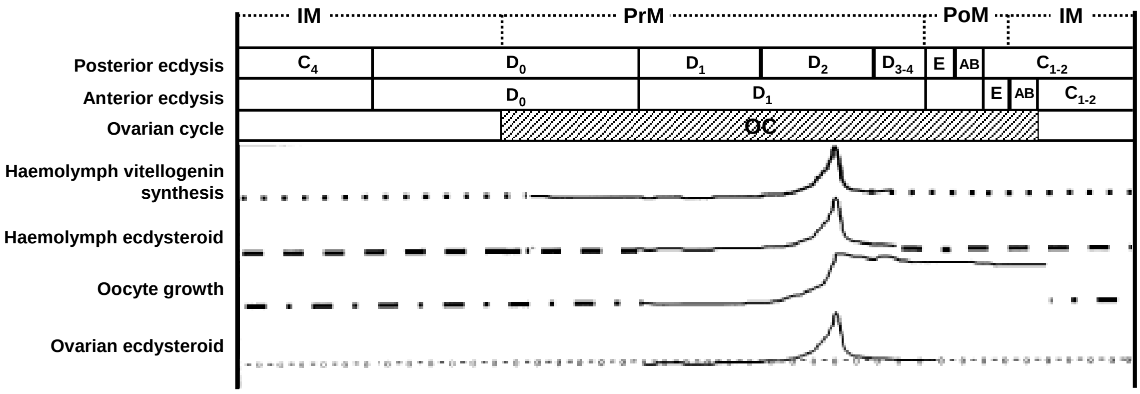

Figure 1.5 - Diagram of the interrelationship between molt and the ovarian cycle in the terrestrial isopod *Oniscus asellus*. Abbreviations: AB, postmolt stages; C_{1-4}, intermolt stages; D_{0-4}, premolt stages; E, ecdysis; IM, intermolt; PrM, premolt; PoM, postmolt; ▨, ovarian cycle; ☐, embryonic cycle. Adapted from Subramoniam (2000).

1.4 Selection of suitable test species for evaluating endocrine disruption

Living organisms are complex structures with a multifaceted mixture of tissue specific and temporal events that are controlled by hormonal mechanisms. As a result, it is important to select relevant endpoints including a variety of life stages. Although there is a vast variety of invertebrates from which to select test organisms, the choice will depend on practical limitations of the test organisms and the availability of measurable endpoints relevant to ED.

Several attributes should be considered when choosing test organisms for addressing the evaluation of potential effects of EDCs (deFur et al., 1999):

- Primary mode of reproduction should be known;
- Organisms should be easily cultured in laboratory;
- Organisms should have short generation times (possibility of full life-cycle and transgenerational exposures);
- Organisms’ size should allow for measurement of hormone levels and other parameters;
- Knowledge of endocrinology;
- Standard methods should be available (e.g. ASTM, OECD, ISO).

Added to this, more general considerations for selecting the test species used for ecological risk assessment (Pinder and Pottinger, 1998) should be taken in consideration:

- Relative sensitivity;
- Ecological importance;
- Ability for *in situ* testing.

Without doubt, terrestrial isopods fulfil the vast majority of these criteria. These animals are abundant in the field throughout the year and are easily hand collected. They are easily reared under laboratory conditions, where they can complete their entire life-cycle (Caseiro et al., 2000). They reproduce sexually and gender and female pregnancy stage are easily distinguished, allowing for uncomplicated study of diverse aspects of their reproductive biology. Their size allows the dissection for organs (e.g. gut, hepatopancreas and gonads), and the detection and quantification of hormones and other biochemical fractions in a single individual. Furthermore, terrestrial isopods are good representatives of saprophytic organisms which carry a key role in organic matter decomposition, a process that is of great importance in soil fertility (Van Vliet and Hendrix, 2004). Although no standard methods have been developed for these organisms (i.e.

ISO, ASTM, OECD), terrestrial isopods have been pointed out as suitable invertebrates for toxicity testing of pollutants in the terrestrial environment (Drobne, 1997), and protocols for toxicity testing are available (Hornung et al., 1998). Information on the endocrinology of terrestrial isopods, albeit limited, has increased and it is relatively well available compared to other invertebrate groups. Having a saltatory growth through a molting process regulated by a vastly studied class of morphogenetic hormones, the ecdysteroids, and having a reproductive process under the control of the same ecdysteroids, makes this organism well suited for ED risk assessment. Methods for vitellogenin synthesis and ecdysteroid titre quantification in woodlice hemolymph are available (Steel and Vafopoulou, 1998), providing useful methods for confirming the endocrine effects of some EDCs.

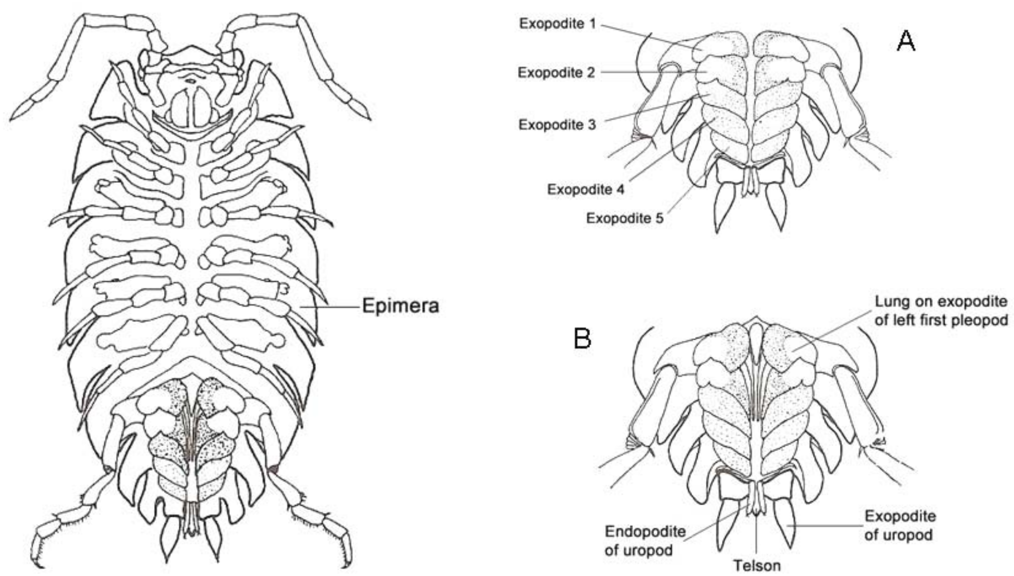

Figure 1.6 - *Porcellio scaber* (Crustacea: Oniscidea) external features in ventral view, A) view of female pleopods and B) view of male pleopods. Redrafted after Hopkin (1991).

Many different endpoints have been studied in woodlice (e.g. *P. scaber*, *P. dilatatus*, *P. leavis*, *Oniscus asellus*). These include survival (e.g. Jansch et al., 2005), growth, food consumption and assimilation (e.g. Loureiro et al., 2006), molt frequency (e.g. Drobne and Strus, 1996), reproduction (e.g. Vink and Kurniawati, 1996), respiration, induction of heat shock proteins and energy reserves (e.g. Knigge and Kohler, 2000), hormone titres (Steel and Vafopoulou, 1998), locomotory behaviour (Engenheiro et al., 2005) and ultrastructural changes in cell structures (e.g. Znidarsic et al., 2003).

P. scaber Latreille (1804), the terrestrial isopod used here (figure 1.6), has been cultured in our laboratory for more than 8 years and these cultures were initiated with animals collected from horse manure in central Portugal. This woodlice is widely spread in Europe and has been extensively used for testing the effects of a wide range of toxicants (Drobne, 1997) including heavy metals (Donker et al., 1993; Farkas et al., 1996; Vijver et al., 2006) and organic compounds (Van Brummelen et al., 1996; Fischer et al., 1997; De Knecht et al., 2001; Engenheiro et al., 2005), both in the laboratory and in the field.

1.5 Conceptual framework of the study

Although several studies have focused on the developmental and reproductive toxicity of natural and synthetic estrogens and androgens in invertebrates (Andersen et al., 1999; Brown et al., 1999; Gray et al., 1999; Duft et al., 2003; Bursztyka et al., 2008; Haeba et al., 2008), there is still discussion on whether these effects are solely the result of general toxicity rather than disruption of endocrine processes (Fukuhori et al., 2005). Unless an evident causal link with ED is provided, one can not speak of ED (deFur et al., 1999; Barata et al., 2004).

Moreover, concerning ED, there is an urgent need to fill the information gap that exists at the environmental level, particularly for the soil ecosystem. The development and validation of tools to provide real evidence of endocrine disruption in invertebrates are vital because these pollutants have the potential to evoke a population decrease. Recovery might be difficult even after the regulation of activities involving these compounds, In this research, the null hypothesis tested was that vinclozolin and bisphenol A do not disrupt the normal development nor compromise the reproduction of the terrestrial isopod *P. scaber*. Potential mechanisms by which these chemicals elicit toxicity were also addressed.

In **chapter two**: “Endocrine disruption in a terrestrial isopod under exposure to bisphenol A and vinclozolin”, the two proposed EDCs were tested for endocrine disrupting activity in *P. scaber*. The effects on male *P. scaber* ecdysteroid levels and on molting behaviour were assessed. 20-hydroxyecdysone titres were assessed after 7, 14 and 28 days exposure to EDC-contaminated soil. In parallel, sex ratios of juveniles exposed for 16 weeks were determined. Effects of Vz and BPA on 20E and its consequences were addressed.

In **chapter three**, “Developmental toxicity of bisphenol A and vinclozolin in a terrestrial isopod”, the effects of the selected fungicide and the industrial chemical on the development of isopods were assessed. Fully mature *P. scaber* male adults and male and female juveniles were exposed to contaminated soil and weighed and photographed weekly. Growth and molt related parameters were followed for 10 weeks for adults and for 16 weeks for juveniles. Developmental toxicity was discussed in the light of endocrine disruption *vs.* classical chronic toxicity.

In **chapter four**, “Reproductive toxicity of the endocrine disrupters, bisphenol A and vinclozolin, in the terrestrial isopod *Porcellio scaber*”, *P. scaber* were paired in contaminated soil. Reproductive parameters monitored were time to reach pregnancy, pregnancy duration, pregnancy and abortion occurrence, number of juveniles per female, juvenile weight, juvenile survival and growth, and reproductive allocation. Isopod reproductive impairment was determined as the sub-chronic effect on adults and lethality of juveniles. The issues of endocrine disruption, elevation of 20E levels and also its relation to vitellogenin were addressed as causal links.

In **chapter five** the “Protein differential expression induced by endocrine disrupting compounds in a terrestrial isopod” was addressed. In this study, a simple and accurate method to extract proteins from the organs of the terrestrial isopod *P. scaber* was developed. Afterwards, protein expression in different organs of male isopods (gut, hepatopancreas and testes) exposed to bisphenol A and vinclozolin was evaluated. The use of this methodology for the analysis of toxicant effects at the molecular level, contributed to unravel the mechanisms underlying the effects already reported at higher organization levels.

In **chapter six**, general conclusions are drawn and future research needs are formulated.

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Chapter 2

Endocrine disruption in a terrestrial isopod under exposure to bisphenol A and vinclozolin

2. ENDOCRINE DISRUPTION IN A TERRESTRIAL ISOPOD UNDER EXPOSURE TO BISPHENOL A AND VINCLOZOLIN

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Abstract

Background, aim, and scope In the past decade there has been an increasing awareness about the possible consequences of human and wildlife exposure to Endocrine Disrupting Compounds (EDCs). Bisphenol A (BPA) and Vinclozolin (Vz) are EDCs which impacts on vertebrates have been largely investigated. Nevertheless, research on invertebrate effects, especially on soil organisms, are still largely underrepresented. This work aims to extend the limited ecotoxicological datasets available and to provide tools to assess the effects of EDCs on the terrestrial species, using *Porcellio scaber* (Crustacea: Isopoda) as a model organism.

Materials and methods Male adult isopods were exposed for 10 weeks to BPA and Ronilan® [containing 50% Vz as active ingredient (a.i.)] at concentrations of 10, 30, 100, 300 and 1000 mg a.i./kg of soil and compared to non-exposed isopods. We studied the effects of these EDCs on molting and total ecdysteroid (20E) concentration. Young, sexually undifferentiated isopods were also exposed to these compounds (Vz: 5, 10, 25, 50 and 100 mg a.i./kg of soil; and BPA: 10, 25, 50, 150 and 300/kg of soil) for 16 weeks and effects on sex-ratio were assessed.

Results Exposure to both chemicals resulted in toxic effects on isopods. Time to first molt was delayed with increasing concentrations of Vz. After 10 weeks exposure to 1000 mg a.i. Vz/kg soil, 100% mortality occurred due to incomplete ecdysis. BPA induced an opposite effect as animals started to molt sooner. Vz significantly increased the 20E titres after 7 and 14 days (LOEC 300 mg a.i. Vz/kg soil) and after 28d of exposure the LOEC value was 100 mg a.i. Vz/kg soil. BPA also induced a 20E concentration increase after 28d of exposure at 10, 300 and 1000 mg/kg soil. In juveniles, we observed a low dose alteration of sex-ratio in BPA-exposed organisms with a skewed ratio of one male per two females, which is in contrast to an almost equal gender distribution in the control. Vz induced no alterations in the sex ratio of isopods.

Discussion Results show that chronic Vz exposure induces a high mortality in *P. scaber.* This is not consistent with other studies describing non-toxicity of fungicides to arthropods. Therefore, it is desirable that toxicity assessment of fungicides is performed via chronic exposure and full life cycle tests. Previously reported low dose responses to BPA in vertebrates are consistent with results of the present study regarding a sex ratio shift induced by low BPA concentrations. Enhanced mortality turned out to be the effect of incomplete ecdysis related to increased ecdysteroids titres. Therefore, "hyperecdysonism" might be a promising endpoint to detect and assess endocrine disruption (ED) in arthropods inhabiting the terrestrial environment.

Conclusions This work reveals that both Vz and BPA disrupt the endocrine function of these important representatives of soil edaphic invertebrates. For the first time, the existence of "low-dose effects" affecting soil invertebrates is reported. Therefore, isopods are suitable organisms for ED assessment and endpoints such as molting, sex ratio or 20E concentration are valuable tools for ecotoxicological studies on hormonally active substances.

Recommendations and perspectives Although the effects observed in the present study have not been induced at environmentally relevant concentrations, synergistic interactions of EDC mixtures present in the environment may well have an impact on arthropods at lower substance concentrations. Additionally, the low-dose sex ratio change demonstrated here confirms the importance of the choice for a large concentration range. The assessment of potential EDCs should consider chronic exposures and life-cycle studies. Although the modes of action of EDCs in many arthropods are fragmentary, parameters like molting impairment, incomplete ecdysis and the determination of hormone titres seem to be suitable biomarkers that should be included as soon as possible in regular surveys for the detection of hormonally active substances.

Keywords 20-hydroxyecdysone • Bisphenol A • Endocrine disruption • Ecotoxicological testing • Hyperecdysonism • Incomplete ecdysis • Low dose effects • Molting • *Porcellio scaber* • Sex-ratio • Terrestrial isopods • Vinclozolin

2.1 Background, aim and scope

There has been a growing concern about the possible consequences of wildlife and human exposure to xenobiotic compounds which are able to modulate the endocrine system, the Endocrine Disruptor Compounds (EDCs). Meanwhile, this issue has also been addressed via several priority research actions and legislation by the European Union (European Commission, 2007), the US Environmental Protection Agency (Harding et al., 2006) and the World Health Organization (Damstra et al., 2002).

Around 95% of known species in the animal kingdom are invertebrates (deFur et al., 1999). Nevertheless, studies on the effects of EDCs on invertebrates are still scarce and, of these, only 10% were conducted with terrestrial invertebrates (Oehlmann and Schulte-Oehlmann, 2003).

Processes under endocrine control, like the molting of arthropods (insects, crustaceans, and of some other minor groups), require periodic loss of the exoskeleton, replacement and hardening with a larger new cuticle. Molting is regulated by ecdysone and related compounds called ecdysteroids (Horn et al., 1966). With the decline of 20-hydroxyecdysone (20E) titres, the hydroxylated and biologically active ecdysteroid, the production of a new cuticle begins. When a threshold value of the ecdysteroid titre is attained, a series of neuropeptides are released causing shedding of the old cuticle by a process termed ecdysis (Subramoniam, 2000). Thus, while classical toxicity testing methodologies are not designed to specifically address ED (e.g., survival, sex ratio, growth rate, molting intervals and success), they can measure adverse integrative effects associated with EDCs.

Isopods are saprophytic organisms that play a key role in the organic matter decomposition in soils. Their importance in the soil system, allied to their easy maintenance in laboratory cultures, sexual reproduction and dimorphism, size of organisms and their distinct molting stages, make isopods ideal model test organisms for ecotoxicological studies (Drobne, 1997). Isopods have been studied after exposure to a wide range of toxicants, including heavy metals (e.g. Loureiro et al., 2009) and organic compounds (e.g. Engenheiro et al., 2005). Most information about the effects of EDCs is available essentially at the toxicological level. At the environmental level the information is scarcer and focused on the effects involving aquatic organisms, thus creating a gap for the soil system. Therefore, this work aims to extend data on the effects of EDCs on the terrestrial species, using *Porcellio scaber* (Crustacea, Isopoda) as a model organism.

In this study, two compounds, listed under the EU priority list of ED (European Commission, 2001), were investigated. Vinclozolin (Vz, 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione; CAS 50471-44-8) is a fungicide which was widely used in agriculture for the production of lettuce, raspberries, beans, onions, vines and others. In Europe, the use of this substance is no longer authorized according to the exclusion of Vz from Annex 1 under EC Directive 91/414. It is a proven endocrine disruptor causing anti-androgenic effects due to its metabolites, which are able to bind to the androgen receptor (Kelce et al., 1994). Vz induces a reduction of the penis and accessory male sex organs and advancement of the sexual response phase in prosobranch snails (Tillmann et al., 2001) and shows epigenetic transgenerational effects (Anway et al., 2006). According to our knowledge, data on accumulation in soil or effects on soil organisms are not available.

Bisphenol A (BPA, 4,4'-dihydroxy-2,2-diphenylpropane; CAS 80-05-7) has been shown to be an oestrogen receptor agonist (Okada et al., 2008) for which anti-androgenic properties were also identified (Lee et al., 2003). It is used to produce epoxy and polycarbonate resins which are employed in the manufacture of a wide range of consumer products, such as food containers, and in medical applications. According to Euling and Sonawane (2005) about 3,200,000 tonnes of BPA are manufactured worldwide each year (based on data for 2005). The presence of BPA in sewage sludge from municipal plants [concentrations of 0.033-36.7 mg/kg (dw)] (Lee and Peart, 2000) and its application to the land to function as soil improver (Furhacker et al., 2000) indicate that contamination of agricultural soils is a real issue, although neither toxicity data for soil organisms nor soil residue data are available.

2.2 Materials and methods

2.2.1 Test organism and culture procedures

Isopods (*Porcellio scaber* Latreille, 1804) came from a culture established in our laboratory for more than 8 years, initiated with animals collected from horse manure in central Portugal. Animals in culture are maintained at 21±1°C, with a 16:8 (light:dark) photoperiod in plastic boxes with a layer of sterile sand and food provided *ad libitum* in the form of oven dried alder (*Alnus glutinosa*) leaves and with 100% relative air humidity.

Pregnant females, with fully-formed marsupium, were placed in a secondary culture until release of mancae. Isopods born within a two-day period were used to start synchronized cultures and later reared individually in polyethylene terephthalate (PET) boxes (Ø60 mm x 30 mm) perforated on the sides to insure ventilation and applying the same substrate and conditions described above.

2.2.2. Chemicals and preparation of soil

An agricultural natural soil from the lower Mondego valley (Portugal) kept in fallow and with no intervention or plant protection products use for the last 5 years was used in these experiments. The soil was oven dried at 60ºC for 48 hours and immediately weighed. Both contaminations were carried out on dry soil.

Bisphenol A (BPA, Merck Schuchardt, Germany, 2,2-bis-(4-hydroxyphenyl)-propane**,** Purity >99%) was dissolved in equal amounts of methanol and mixed with soil at 10, 30, 100, 300 and 1000 mg/kg dry soil for experiments with adults. For juvenile testing, toxicant concentrations were 10, 25, 50, 100 and 300 mg/kg dry soil. After BPA spiking, soil was left to dry under a fume hood for 12 hours. Moisture content was subsequently adjusted to 20% (v/w) with distilled water. As a control, moisture content was adjusted as described above. A solvent control (0^+) was prepared with the same volume of methanol without BPA, then left to dry and moisture content adjusted as described above.

Vinclozolin (Ronilan® containing 50% Vz as active ingredient (a.i.); BASF AG**,** Germany) dissolved in water, was mixed with soil at 10, 30, 100, 300 and 1000 mg a.i./kg dry soil for adult experiments and at 5, 10, 25, 50 and 100 mg a.i./kg dry soil for juvenile testing. The soil moisture content was then adjusted to 20% (v/w) with distilled water**.**

Chemical analysis of Vz and BPA treatments concentration were performed and were within ± 5% of the nominal concentrations. The results are presented in terms of the nominal values.

2.2.3 Organism exposure

2.2.3.1 Adult isopod testing (molting and 20-hydroxyecdysone)

All experiments were performed at the same temperature, photoperiod and relative humidity conditions as described above for *P. scaber* cultures. Isopods were taken from the lab culture weighing 20±1 mg. Only male adults were used to ensure that female reproductive traits did not influence the endpoints assessed.

Thirty-two animals, at intermolt stage, per treatment were randomly and individually placed in PET boxes (Ø100 mm x 50 mm) filled with 60 g of spiked agricultural soil and four Ø10 mm alder leaf discs providing the same shelter area and food. Leaf discs were added weekly, when necessary, in order to maintain food *ad libitum*. Molting stage was determined at weekly intervals for 10 weeks.

2.2.3.2 Juvenile isopod testing (sex ratio determination)

Fifty juveniles per treatment, weighing between 4 and 5 mg, were separated before secondary sexual characters occurred, insuring also virginity of the animals and, thus, preventing reproductive processes during the experimental period. These animals were randomly and individually placed in PET boxes (Ø60 mm x 30 mm) filled with 30 g of spiked agricultural soil and two Ø10 mm alder leaf discs, which provided the same shelter area and food. Leaf discs were added weekly, when necessary, in order to maintain food *ad libitum*. All juvenile isopods were exposed to toxicants before differentiation of the secondary sexual characters. After the 16-week experimental period, sex was determined, and the sex-ratio of test organisms was calculated for each treatment group. Gender was determined by checking male differentiation of the endopodites on the second pair of pleopods formed into copulatory legs.

2.2.4 Measurements, photographs and image analysis

P. scaber, as all crustaceans, has saltatory growth, increasing size after every ecdysis. An increase of cuticle size is a reliable sign that they have molted. This, together with the verification of calcium deposits on thoracic sternites (Zidar et al., 1998), guarantees the reliable identification of molting occurrence. The medium segment of twelve adults per treatment was photographed under stereo dissecting microscope for posterior digital image analysis using Leica Qwin, Image Processing and Analysis Software® for cephalothorax width (CW) measurements. Occurrence of molt was inferred from the difference of size in consecutive measurements.

2.2.5 Ecdysteroid analysis

After one, two and four weeks, eight intermolt individuals per treatment were analysed for ecdysteroid quantification. Isopods were frozen in liquid N_2, lyophilized (Snijders Scientific, type 2040 lyophilizer, Tilburg, The Netherlands) and dry weights were measured. Afterwards, individuals were homogenized in methanol and prepared according to the method of Block et al. (2003). Ecdysteroid measurements were carried out with 20-hydroxyecdysone EIA kits from Cayman Chemical Company (Ann Arbor, MI, USA) according to the manufacturer's instructions. The colorimetric reaction was measured at 414 nm using the Labsystems Multiskan EX plate reader (Helsinki, Finland). Ecdysteroid titre of each sample was determined by comparison of sample absorbances with the 20-

hydroxyecdysone standard curve and expressed as pg of ecdysone equivalents/mg dw (Polgar et al., 1996).

2.2.6 Statistical analysis

LC50 values and corresponding 95% confidence limits were determined by the Probit Analysis Method (Finney, 1971). All data were checked for normality and homoscedasticity. One way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means were employed to determine significant differences relatively to the control treatment. Significant differences for sex-ratios relatively to the control treatment were assessed using the G-test for goodness-of-fit for which two-tailed p values were calculated. Where applicable, results are presented as mean ±SE. For all statistical tests, the significance level was set at $P \leq 0.05$. All calculations were performed with SigmaStat (Systat Software Inc., 2006).

2.3 Results

2.3.1 Lethality of Vinclozolin and Bisphenol A

After four weeks exposure of adult *P. scaber*, LC_{50} values were higher than 1000 mg a.i. Vz and BPA/kg soil (37.5% mortality at this highest concentration for both test chemicals). After ten weeks of exposure, 1000 mg BPA/kg soil killed 50% of the animals [LC_{50} (95% CI) was 910 (163–1,658) mg/kg soil]. Exposure to 1000 mg a.i. Vz/kg soil led to 100% mortality and half of the adult isopods died at 300 mg a.i. Vz/kg soil, resulting in an LC_{50} (95% CI) of 298 (150–447) mg/kg soil. The LC_{50}s for the juvenile isopods were higher than the highest concentrations tested for both chemicals, even after 16 weeks.

2.3.2 Developmental toxicity of Vinclozolin and Bisphenol A

The majority of organisms in control conditions molted in the first two weeks, 25% in week 1 and 50% in week 2. About 87.5% of isopods exposed to Vz (10 mg a.i. Vz/kg soil) molted in the first two weeks (Fig. 2.1A). Molt seemed to be drastically delayed with increasing concentration of the contaminant. In the highest concentration tested, 1000 mg a.i. Vz/kg soil, only 12.5% of animals molted in the first two weeks, 50% of them only molted in the second month.

Isopods kept in soil treated with the BPA's solvent, methanol, experienced an extremely delayed molt with 87.5% of first molts being observed only in the third week (Fig. 2.1B). This severe molt delay as a consequence of solvent exposure seems to be mitigated by increasing levels of BPA in a concentration-dependent way. At the BPA concentration of 1000 mg/kg soil a quarter of the animals molted in the first week and around 63% in the first 14 days, resembling the control conditions.

2.3.3 Ecdysteroids titres

The levels of 20E during the intermolt period were determined for adult male isopods. An average value of 29.7 ±4.3 pg ecdysone equivalents/mg dw during intermolt reaching a peak concentration of 79.4 ±3.8 pg ecdysone equivalents/mg dw during premolt was observed for the control group. Vz induced a time and concentration-dependent increase of ecdysone equivalents after 7, 14 and 28 days of exposure (Fig. 2.2A). The level of 20E increased after 7 and 14 days exposure with a NOEC of 100 mg a.i. Vz/kg soil (ANOVA, Dunnett's test, $F_{5.39}$= 10.553, P< 0.001 and $F_{5.38}$= 5.757, P< 0.001, respectively). Furthermore, after 28 days of exposure to the fungicide, 20E titres were elevated compared to controls, being statistically significant from 100 mg a.i. Vz/kg soil onwards (ANOVA, Dunnett's test, $F_{5.38}$= 6.925, P< 0.001).

In BPA contaminated soil (Fig. 2.2B), although ANOVA detected differences of 20E titres of 14 days exposed groups compared to solvent control (ANOVA, Dunnett's test, $F_{5.35}$= 4.752, P< 0.001), the post hoc test was not able to distinguish any differences. With extended exposure (28 days), 20E titres increased and were significantly different for animals exposed to the lowest (10 mg BPA/kg soil) and the highest concentrations (300 and 1000 mg BPA/kg soil), when compared to the solvent control (ANOVA, Dunnett's test, $F_{6.39}$= 5.270, P< 0.001).

2.3.4 Gender ratio in isopods exposed to Vinclozolin and Bisphenol A

The sex ratio found under control conditions (number of males/number of females) was 0.95, i.e., nearly one male per every female (Fig. 3). Exposure to the fungicide Vz did not alter this ratio (Fig. 2.3A). A skewed sex ratio was observed for BPA (Fig. 2.3B). Gender ratio in the lowest concentration (10 mg BPA/kg soil) changed in favour of females to a ratio of one male per two females (G test, G= 5.303, df 1, P= 0.021). Higher BPA concentrations in the soil resulted in the same effect, although differences were not statistically significant.

2.4 Discussion

To date, no toxicity data of Vz and BPA has been presented for isopods. Vz and BPA revealed very low mortality rates for the 28 days adult exposure test. When extending the exposure period to ten weeks we found a severe increase of mortality in Vz exposed treatment groups. This is not consistent with the common low acute toxicity to arthropods shown for other fungicides (Jansch et al., 2005; Haeba et al., 2008). Vinclozolin has a low to moderate persistence in soil (IUPAC, 2006), with reported half-lives from 28-43 days in the laboratory up to 34-94 days in the field (U.S. EPA, 1991; IUPAC, 2006). Despite this low-persistency of Vz, this increase of mortality with exposure time may be due to the increased concentration of its' major metabolites, M1, M2 and 3,5-DCA, which are reported to be more toxic than the parent compound (Kelce et al., 1994) and with half-lives ranging from 179 to >1000 days (U.S. EPA, 2000). Therefore, it is desirable that toxicity assessment of fungicides is performed via chronic exposure and full life-cycle tests.

Molting has been considered as an important endpoint by several authors and it is known to be affected by various substances and conditions, such as temperature and nutritional state of the organism (Weis et al., 1987; Drobne and Strus, 1996). Although development and molting parameters can not be considered exclusive in the assessment of hormonally active substances, they can measure adverse integrative effects associated with ED when a direct causal link with hormonal disruption is proven (deFur et al., 1999).

The solvent used in the experiments, methanol, introduced a large difference in the time to reach molt, probably by altering the physical properties of the soil (water holding capacity, organic matter availability, and others), thereby creating stressful situations that have affected the molt process. Nevertheless, there was a clear reduction in the time taken to first molt caused by the exposure to BPA when compared to the solvent control treatment.

The xenoestrogen BPA induced a sex-ratio shift favouring female isopods. In prosobranch snails, it has been reported that female-biased sex ratios were caused by the phenomenon of feminization induced by BPA (Oehlmann et al., 2000). Nevertheless, the parsimony principle withstands the simpler and straightforward explanation that a skewed sex ratio could simply be due to differential lethality of one of the genders to the toxicant (Callahan and Weis, 1983). This issue could be tackled in the future by performing gonad histopathology in exposed isopods and assessing abnormalities associated with masculinised females or feminised males, or by identifying genetic sex markers that can be used to compare genetic sex with individual phenotypic secondary sexual characters.

Although other than endocrine modes of action can not be excluded, the classical concentration response paradigm was replaced by a sex ratio shift and increased molting hormone levels at low concentrations, which is in agreement with several studies where lower concentrations of BPA induced larger effects (Welshons et al., 2006; Izumi et al., 2008). Furthermore, this is backed by the US EPA expert panel which confirms these exclusively low-concentration effects of endocrine-mimicking chemicals (Kaiser, 2000). In a *Drosophila melanogaster* B_{II} cell *in vitro* assay, BPA was able to compete with ecdysteroids for the ligand binding site on the receptor complex (Dinan et al., 2001). Planello et al. (2008) demonstrated that BPA acts as an ecdysone-mimetic compound and up-regulates the levels of ecdysone receptor (EcR) in *Chironomus riparius* (midge) cells. This competition for the receptor might be responsible for the 170% increase of the 20E levels in the lowest concentration tested (10 mg BPA/kg soil) and over 148% increase for the two highest concentrations (300 and 1000 mg BPA/kg soil) after 28 days of exposure. The reason for this hormone balance impairment remains speculative and, as argued by Mu et al. (2005) and supported by the current work, the precise mechanism of toxicity does not involve depletion of endogenous ecdysteroid levels. Nonetheless, one may hypothesize that ecdysone receptor binding feedback mechanisms might not have been triggered due to unspecific binding, and that production/elimination mechanisms might have been unbalanced leading to increased 20E titres. Vz, in contrast to BPA, delayed the first molt, with highest mortality observed only at higher concentrations as a consequence of incomplete ecdysis during this first molt. Weis et al. (1987) also noted significant mortality close to the time of ecdysis at higher concentrations of diflubenzuron in the fiddler crab *Uca pugilator* and Baldwin et al. (2001) observed mortality at this molting stage in *Daphnia magna* exposed to 20E and ponasterone A.

Prior to ecdysis, ecdysteroid titres raise and a sudden decrease of these levels trigger the completion of exuviation (Subramoniam, 2000). It has been reported that the phenomenon of hyperecdysonism - a higher basal level of 20E - overrides this normal precipitation of ecdysteroids preventing animals from exuviating probably by impairing the release of an exuviation factor (Bodar et al., 1990). In our study, the mortality due to incomplete ecdysis may be related to the high levels of ecdysteroids found with increasing concentrations of Vz. Due to the lack of exuviation factor animals suffered from delayed or incomplete molts, cumulating in mortality at higher substance concentrations. Therefore, incomplete ecdysis and hyperecdysonism may become potential bioindicators to detect disruption of ecdysteroid function.

Vz is a proposed anti-androgen that has been shown to interfere with steroid hormone homeostasis. Hormonal balance plays a decisive role in both primary sexual determinations and acquisition and maintenance of secondary sex characteristics in adults, thus establishing gender during the pre and neonatal period (Colborn et al., 1993; LeBlanc et al., 1997). Signs of demasculinisation and female-biased populations were confirmed by several authors. Haeba et al. (2008) found a 2-fold decrease in the number of male neonates in *D. magna* exposed to 1mg Vz/L and in molluscs exposed to Vz, where the lengths of the penis and of accessory male sex organs (e.g. penis sheath, prostate) were reduced (Tillmann et al., 2001). Nevertheless, in our experiments, Vz exposure had no significant effects on the gender ratio in isopods.

EDCs acting at the level and time of sexual differentiation in an invertebrate may skew sex ratios in favour of males (Matthiessen and Gibbs, 1998; Olmstead and LeBlanc, 2003) or females (Oehlmann et al., 2000; Haeba et al., 2008) depending on the oestrogenic or androgenic mechanisms involved. The study of sex ratios of natural populations might provide a simple and robust indication of EDC activities in the environment provided that the normal gender distribution of undisturbed populations is known. Also, the characteristic patterns of ecdysteroid concentration in isopods during the molt cycle provide a measurable marker of hormonal function. As soon as baseline data have been established for control populations, it is possible that measurements of changes in ecdysteroid titres can be applied as a routine tool for biomonitoring studies, including the detection of hormonally active substances.

2.5 Conclusions

Vinclozolin and Bisphenol A are well-documented endocrine disruptors in vertebrates and have already been investigated in some aquatic invertebrates. The results from the present study demonstrate that both the fungicide Vinclozolin and the industrial chemical Bisphenol A cause endocrine disruption in *P. scaber* with an ecdysteroid up-regulation resulting in molting disturbances. Since endocrine-mediated chronic effects were identified at much lower concentrations than those showing acute toxic effects on isopods, molting impairment, incomplete ecdysis and hormone levels are easy and suitable biomarkers that may provide further valuable endpoints for EDCs identification and characterisation.

2.6 Recommendations and perspectives

Isopods like *P. scaber* combine the features of continuous growth, through a molting regime, with a terrestrial mode of life, making them suitable sentinel species to detect hormonally active soil pollutants. After the maximum recommended application rate of Ronilan®, the concentration of vinclozolin in the soil is 1 mg a.i./kg (assuming that 70 % of the fungicide will reach the surface and is homogeneously distributed over the top 5 cm soil layer and the soil bulk density is 1.4 kg/dm^3). Vinclozolin has a low to moderate persistence in soil, with reported half-lives from 28-43 days in the laboratory up to 34-94 days in the field (U.S. EPA, 1991; IUPAC, 2006). The only significant route of BPA to the terrestrial environment is through the application of sewage sludge from municipal plants [concentrations of 0.033-36.7 mg/kg (dw)] (Lee and Peart, 2000) as soil improvers. The half-life for bisphenol-A in soil was calculated as from 3 days (Fent et al., 2003) up to 37.5 days (based on modelled half-life in water) (Environment Canada, 2008). Despite the concentrations resulting in adverse effects in the present study are well above these expected environmental levels and, at a first glance, it seems that Vinclozolin and Bisphenol A pose no threat to natural populations of terrestrial arthropods, caution should be taken in ecological risk assessments since chemicals that interfere with ecdysteroidal activity are common in the environment, and effects due to this class of compounds might become more enhanced in mixtures than at individual concentrations previously classified as safe.

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Figures

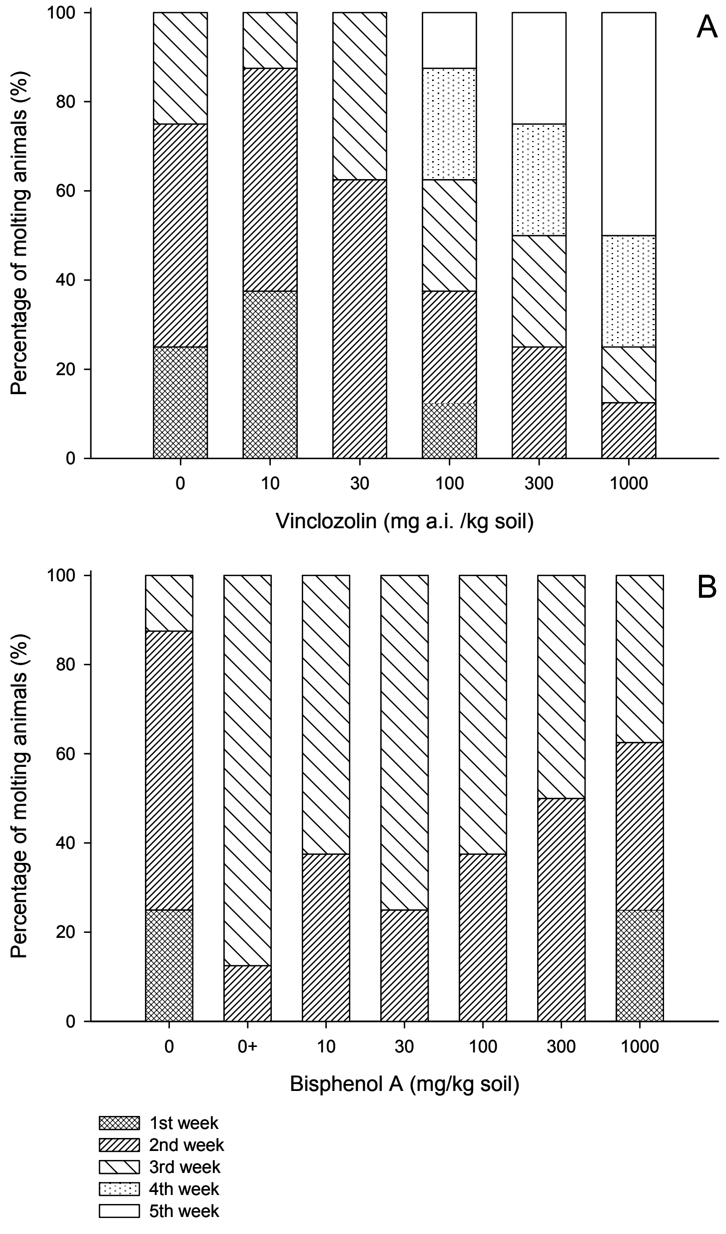

Figure 2.1 - Distribution of first molt per week of *Porcellio scaber* after exposure to soil treated with A) Vinclozolin and B) Bisphenol A. Data are presented as percentage of animals that molted during each period.

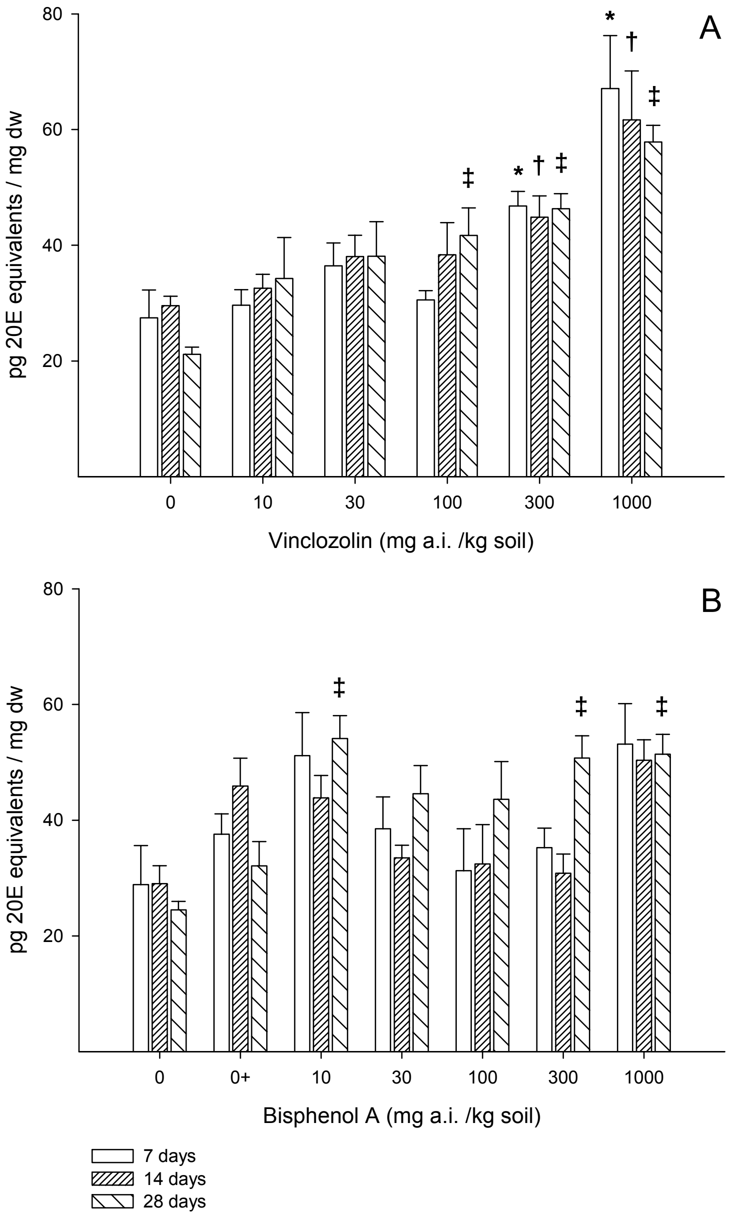

Figure 2.2 - Ecdysteroid titres of *Porcellio scaber* following 7, 14 and 28 days exposure to A) Vinclozolin or B) Bisphenol A. Significant differences from control or solvent control ($p<0.05$, ANOVA, Dunnett's test): * 7 days; † 14 days; and ‡ 28 days.

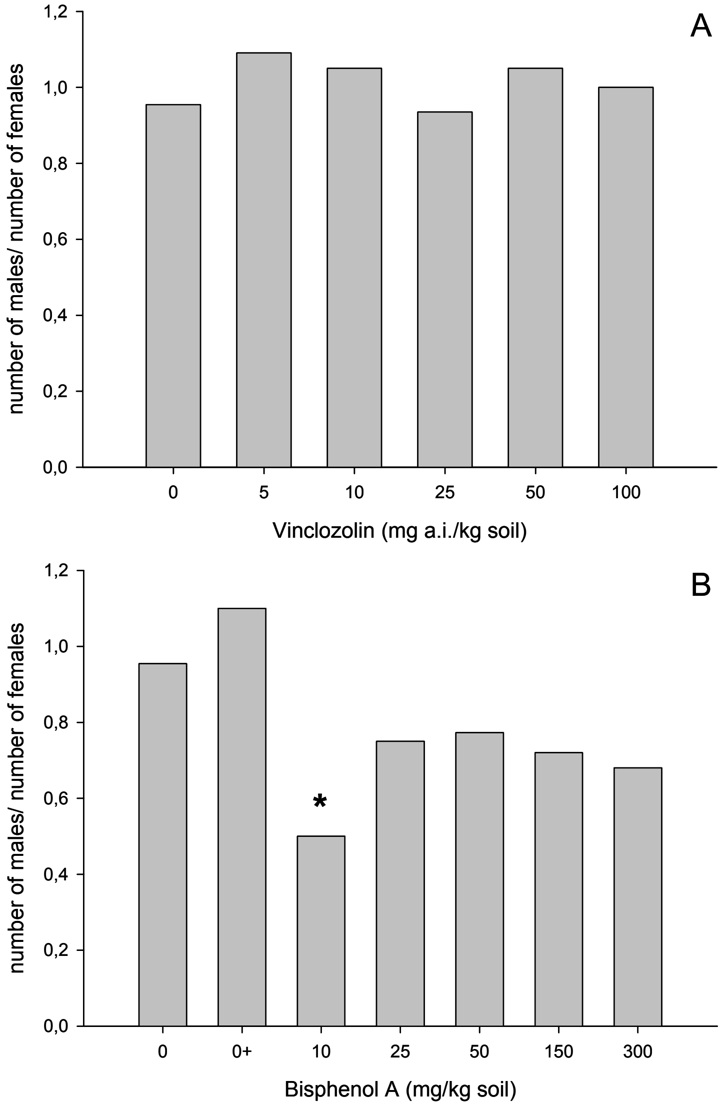

Figure 2.3 - Sex ratio of *Porcellio scaber* after exposure to soil treated with A) Vinclozolin and B) Bisphenol A, represented as the number of males/ number of females. An asterisk indicates a significant difference from the solvent control (P ≤0.05, G-test).

Chapter 3

Developmental toxicity of the endocrine disrupters bisphenol A and vinclozolin in a terrestrial isopod

3. DEVELOPMENTAL TOXICITY OF THE ENDOCRINE DISRUPTERS BISPHENOL A AND VINCLOZOLIN IN A TERRESTRIAL ISOPOD

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Abstract

Studies of the effects of endocrine disruptor compounds (EDCs) on invertebrates are still largely underrepresented. This work aims to fill this gap by assessing the effects of bisphenol A (BPA) and vinclozolin (Vz), on the terrestrial isopod *Porcellio scaber*. Male adult and sexually undifferentiated juvenile isopods were exposed to the toxicants. Effects on molting regime and growth were investigated, independently for males and females after sexual differentiation. Both chemicals elicited developmental toxicity to *P. scaber* causing overall growth reduction, although BPA induced molting while Vz delayed it. Though the LC_{50} values for juvenile and adult survival were fairly similar, juveniles revealed an increased chronic sensitivity to both chemicals and females were most sensitive to BPA. We recommend using adults, juveniles, females and males and a large range of toxicant concentration in order to provide valuable information regarding differential dose-responses, effects and threshold values for EDCs, which may impair population dynamics.

Capsule: EDCs Bisphenol A and Vinclozolin elicit overall developmental toxicity.

Keywords: Bisphenol A • Vinclozolin • *Porcellio scaber* • Endocrine disruption • Terrestrial isopods

3.1. Introduction

Endocrine disruptor compounds (EDCs) are a structurally diverse group of chemicals that may adversely affect the health of humans and wildlife, both at individual and population levels, by interacting with the endocrine system (Porte et al., 2006). They include organic chemicals used heavily in the past, specifically in industry and agriculture, such as polychlorinated biphenyls and organochlorine pesticides. EDCs also include chemicals currently used, such as plasticizers and surfactants. Among these, Bisphenol A (4,4'-dihydroxy-2,2-diphenylpropane; CAS 80-05-7) and Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione; CAS 50471-44-8] are included in the compounds priority list proposed by the EU (European Commission, 2001).

Vinclozolin (Vz) is a fungicide widely used in agriculture for the production of lettuce, raspberries, beans, onions, vine and others (Price et al., 2007). In Europe, the use of this substance is no longer authorized according to the exclusion of Vinclozolin from Annex 1 under EC Directive 91/414. It is a proven EDC causing anti-androgenic effects due to its metabolites (Bursztyka et al., 2008), which are able to bind to the androgen receptor (Anway et al., 2005). It induces Leydig cell tumors (Kavlock and Cummings, 2005), reduces ejaculated sperm numbers and prostate weight in male rats (Monosson et al., 1999), leads to a delayed puberty, demasculinisation and feminization of male offspring in rats (Gray et al., 1999). It induces as well the reduction of the penis and accessory male sex organs and advancement of the sexual repose phase in prosobranch snails (Tillmann et al., 2001).

Bisphenol A (BPA) is used to produce epoxy and polycarbonate resins which are employed in the manufacture of a wide range of consumer products, such as food containers, and in medical applications (Crain et al., 2007). It has been shown to be an oestrogen receptor agonist (Okada et al., 2008) for which anti-androgenic properties were also identified (Lee et al., 2003). BPA is known to act as a teratogen (Crain et al., 2007) and may also lead to the alteration of sex determination and gonadal function (Crain et al., 2007). An enlargement of the accessory pallial sex glands, gross malformations of the pallial oviduct section resulting in an increased female mortality, and a massive stimulation of oocyte and spawning mass ("superfemale" syndrome), was reported in the BPA exposed freshwater ramshorn snail *Marisa cornuarietis* and the marine gastropod *Nucella lapillus* (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2004). The presence of BPA in sewage sludge from municipal plants [concentrations of 0.033-36.7 mg/kg (dw)] (Lee and Peart, 2000) and its use as soil fertilizer (Furhacker et al., 2000) indicate that contamination of agricultural soils is a real issue.

From the many reports that have been published on endocrine disruption (ED), the majority focus on vertebrates (mainly human) and only a minor fraction investigate the effects of ED in invertebrates, although these represent the large majority of all species described (deFur et al., 1999). From the studies reported in the literature, only 10% were conducted with terrestrial invertebrates (Oehlmann and Schulte-Oehlmann, 2003). This value is almost residual when restricting these studies to edaphic fauna.

By interfering with the syntheses, secretion, transport, action or elimination of natural hormones in the organism, EDCs cause effects that are seen at various levels from homeostatic mechanisms, reproduction, development, behaviour, immunology, to nervous impairment (Rodriguez et al., 2007). These interferences may ultimately lead to population declines and even to local extinctions.

Processes under endocrine regulation, like molting of arthropods (like insects and crustaceans) involve a combination of molecular, physiological, and behavioural actions (LeBlanc, 2007). Altering any of these processes may disrupt many physiological processes even if ED is not involved. Thus, while classical toxicity testing methodologies to assess acute and/or chronic effects are not specifically designed to evaluate ED (e.g., growth rate and molt time and success), they can measure adverse integrative effects associated with EDCs (deFur et al., 1999).

Isopods are saprophytic organisms that carry a key role in the organic matter decomposition in soils. Their importance in the soil system, allied to their easy maintenance in laboratory cultures, sexual reproduction and dimorphism, size of

organisms and their easily distinct molting stages, make isopods ideal model test organisms for ecotoxicological studies (Drobne, 1997).
Since information on the effects of EDCs is scarce and focused on the effects on vertebrates and some aquatic invertebrates, there is a gap for the soil compartment. This work aims to contribute to fill that gap, by generating data on the effects of EDCs on the terrestrial system, using the terrestrial isopod, *Porcellio scaber*, as a model organism.

3.2. Materials and Methods

3.2.1 Test species and culture procedures

The isopods (*Porcellio scaber* Latreille, 1804) came from a culture established in our laboratory for more than 8 years and initiated with animals collected from horse manure in central Portugal. Cultures were maintained in plastic boxes with a layer of sterile sand and fed *ad libitum* with alder (*Alnus glutinosa*) leaves, at 21 ±1ºC, 16:8 (light:dark) photoperiod and 100% relative air humidity.
Pregnant females, with fully-formed marsupium, were placed in a secondary culture. Mancae born within the same day were used to start synchronized cultures. Once they attained 4-5mg of weight they were placed individually in separate plastic boxes (Ø60 mm x 30 mm) perforated on the sides to insure ventilation and under the same conditions described above.

3.2.2. Chemicals and preparation of soil

A natural soil from the lower Mondego valley (central Portugal) kept in fallow for the last 5 years was used in these experiments. Soil parameters are summarized in table 3.1. The soil was oven dried at 60ºC for 48 hours and immediately weighed. All contaminations were carried out on dry soil.
Bisphenol A (Merck Schuchardt, Germany, purity >99%) was solubilised in equal amounts of methanol and mixed in with soil at 10, 30, 100, 300 and 1 000 mg/kg

dry soil for experiments with adults. For juvenile testing, toxicant concentrations were 10, 25, 50, 100 and 300 mg/kg dry soil. After BPA addition, the solvent was allowed to evaporate by placing the soil under a fume hood for 12 hours. Subsequently, moisture content was adjusted to 20% (v/w) with distilled water. A solvent control was prepared in the same way, using the same volume of methanol without BPA, then left to dry and moisture content adjusted as described above (represented as 0^+ from this point forward). A control was prepared with water added to dry soil.

Vinclozolin (Ronilan® 50% active ingredient (a.i.); BASF AG, Germany) with water as vehicle, was mixed in with soil at 10, 30, 100, 300 and 1 000 mg a.i./kg dry soil for adult experiments and at 5, 10, 25, 50 and 100 mg a.i./kg dry soil for juvenile testing. The soil moisture content was then adjusted to 20% (v/w) with distilled water. A control was prepared with water added to dry soil.

Chemical analyses of test concentrations were made at the Terracon Laboratorium für Umwelt und Pestizidanalytik GmbH (Jütterborg, Germany). Vz contaminated soil samples (10g) were extracted using acetonitrile (Carl Roth GmbH, Karlsruhe, Germany) in an ultrasonic bath at 40ºC for 30 min. Vz concentration determination was performed in a GC-MS system (Varian, USA) including a 3400 GC ion-trap MS-detector Saturn II, Autosampler 8200 8200cx and Saturn II GC-MS software with NIST 2002 MS-library. BPA contaminated soil samples (15g) were extracted in Soxhlet-apparatus with methanol for 3 hours. The extract was evaporated to 10 ml by rotary vacuum evaporator (40ºC). BPA concentrations were determined by UV detection at 230 nm using a HPLC-PDA system (Shimadzu, Japan) equipped with a C_{18} column (5μm) 2 HPLC pumps model LC-10ADvp, an Autosampler SIL-10ADvp, a column oven CTO-10ASvp, and a photo diode array-detector (PDA) SPD-M10Avp.

Analytical values were within ± 5% of the nominal concentrations. The results are therefore presented in terms of the nominal values.

3.2.3 Organism exposure

All experiments were performed at the same temperature, photoperiod and relative humidity conditions described above for *P. scaber* culturing.

3.2.3.1 Adult isopod growth test

Isopods were taken from the lab culture weighing 20±1 mg. Only male adults were use to ensure that female reproductive traits did not influence the parameters assessed.

Animals were checked for molting stage and only animals at intermolt stage were used (Zidar et al., 1998), ensuring that all organisms were in the same vulnerability stage.

Twelve animals per treatment were randomly and individually placed in polyethylene terephtalate (PET) boxes (Ø100 mm x 50 mm) filled with 60 g of spiked soil and four Ø10 mm alder leaf discs providing the same shelter area and food across replicates. Leaf discs were added weekly, when necessary, in order to keep food in excess. Growth was monitored at weekly intervals for 10 weeks as described below.

3.2.3.2 Juvenile isopod growth test

Synchronized juvenile isopods weighing between 4 and 5 mg and with similar cephalothorax widths (between 1.0 and 1.1 mm) were used. Thirty animals per chemical concentration were separated before differentiation of secondary sexual characters, to insure virginity of the animals and thus preventing reproductive processes during the experimental period. The animals were randomly and individually placed in PET boxes (Ø60 mm x 30 mm) filled with 30 g of spiked soil and two Ø10 mm alder leaf discs, which provided same shelter area and food across replicates. Leaf discs were added weekly, when necessary, to maintain food in excess. Growth was determined at weekly intervals for 16 weeks as described below and gender was determined by the end of the experimental period.

3.2.4 Measurements, photographs and image analysis

All crustaceans have saltatory growth, increasing size after every molting event. An increase of cuticle size is a reliable sign that they have molted. This, together with the verification of calcium deposits on thoracic sternites (Zidar et al., 1998), makes it a strong tool for the identification of molting occurrence. Twelve adult and 30 juvenile isopods per treatment where weighed weekly and the medium segment was photographed under a stereo dissecting microscope for posterior image analysis using Leica Qwin, Image Processing and Analysis Software®. Cephalothorax width (CW) was then measured. Growth per molting interval and the occurrence of molt was then inferred from difference of size in consecutive measurements.

3.2.5 Statistical analysis

All data were checked for normality and homoscedascity. One way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means were employed to determine significant differences relatively to control treatment. Where applicable, results are presented as mean ±SE. For all statistical tests the significance level was set at $P \leq 0.05$. All calculations were performed with SigmaStat (Systat Software Inc., 2006).

3.3. Results

3.3.1 Effects of Vinclozolin and Bisphenol A on adult *Porcellio scaber*

No significant differences in growth per molt were found among Vz treatments and control (ANOVA, Dunnett's test for first molt: $F_{5.32}$= 0.341, P= 0.884; second molt: $F_{4.29}$= 1.892, P= 0.139; and third molt: $F_{3.18}$= 0.778, P= 0.522). Due to 100% mortality at the highest concentration after 10 weeks of testing, we could only assess the growth after the first molt for this treatment.

After 10 weeks, control isopods gained 83% of mass and showed an increase of 24% in size (fig.3.1 A). Those exposed to lower concentrations of Vz tended to grow more than the controls, while a decreased growth with increasing concentration was visible from 30 mg a.i. Vz/kg soil onwards. The increase of the cephalotorax width (CW) was lower and significantly different from controls exposed to 300 mg a.i. Vz/kg soil (ANOVA, Dunnett's test, $F_{4.29}$= 2.941, P= 0.037), but weight was not affected.

As for BPA exposed isopods, there was no difference in growth after the first molt (ANOVA, Dunnett's test, $F_{5.37}$= 2.284, P= 0.07), but at concentrations of 10 and 1 000 mg BPA/kg soil growth was significantly decreased for the second and third molts (ANOVA, Dunnett's test, $F_{5.35}$= 3.137, P= 0.018 and $F_{5.33}$= 3.222, P= 0.018 respectively) (fig.3.2). Isopod mass gain after ten weeks decreased with increasing exposure to BPA (fig.3.1B) with a NOEC of 300 mg BPA/kg soil (ANOVA, Dunnett's test, $F_{5.33}$= 3.306, P= 0.018). No effects were seen on CW increase.

3.3.2. Effects of Vinclozolin and Bisphenol A on juvenile *Porcellio scaber*

Juvenile growth and molting regime were monitored for 16 weeks. Around 60% of non-exposed female individuals molted mostly in the first week (fig.3.3B). From the concentration of 25 mg a.i. Vz/kg soil onward there seemed to be a decrease of animals molting in the first week and in treatments with 50 and 100 mg a.i. Vz/kg soil most animals molted in the second week (55 and 67% respectively). Males showed the same response as females, but only at the highest concentration, with

38% of the individuals molting in the first week (fig.3.3A). The total number of molts during the 16 week testing period was determined. The average number of molts for males and females was 3.6 and 3.9 respectively in the control (fig.3.4). The number of molts decreased with increasing concentrations of the fungicide, both for males and females and was significantly different at 100 mg a.i. Vz/kg soil (ANOVA, Dunnett's test, $F_{5.59}$= 2.628, P= 0.033 and $F_{5.68}$= 2.659, P= 0.030, respectively) (see figure 3.4).

Both size and mass gain were significantly impaired by Vz in both genders (fig.3.5A and B). Isopod CW gain decreased with increasing toxicant concentrations, and was significantly different at Vz concentration of 100 mg a.i./kg soil compared to isopods in non-treated soil [males and females with 14% and 16% reduction respectively (ANOVA, Dunnett's test, for males: $F_{5.80}$= 2.647, P= 0.045; and females: $F_{5.61}$= 2.422, P= 0.045)]. Isopod weight gain also decreased with increasing concentrations of the fungicide with a NOEC of 50 mg a.i. Vz/kg soil for males and females (ANOVA, Dunnett's test, $F_{5.80}$= 2.585, P= 0.036 and $F_{5.61}$= 2.565, P= 0.036 respectively).

Most juvenile control isopods molted in the first week (59% of females and 55% of males) and 23% of females and 27% of males molted in the second week. Exposure to BPA increased the number of woodlice that molted during the first week, with 85% of the isopods undergoing molting in this period at 300 mg BPA/kg dry soil (fig.3.3C and D).

There is a trend for BPA to reduce time to first molt, but no statistically significant differences in the number of molts were observed between treatments during the 16 week experimental period (ANOVA, Dunnett's test, $F_{5.52}$= 4.052, P= 0.477 for males and $F_{5.52}$= 4.052, P= 0.255 for females). Weight and size gain were reduced with increasing concentrations of BPA with a LOEC value of 25 mg BPA/kg soil for size gain (ANOVA, Dunnett's test, $F_{5.52}$= 4.052, P= 0.004) and for weight gain (ANOVA, Dunnett's test, $F_{5,52}$= 5.537, P< 0.001) for males (fig.3.5C and D). Although a significant effect was found on female size and weight gain compared to the solvent control (ANOVA, Dunnett's test, $F_{5.64}$= 3.303, P= 0.012 and $F_{5.64}$= 6.300, P< 0.001, respectively), an irregular concentration response pattern was observed for the growth of females exposed to BPA (fig.3.5C and D).

3.4. Discussion

In a previous work, it was shown that male adult *P. scaber* molt was drastically delayed with increasing concentration of Vz while BPA exposure speeded up the occurrence of the molting event (Lemos et al., 2009a). These molting perturbations were attributed to an increase of the endogenous molting hormone, 20-hydroxyecdysone concentration, which enabled to suggest a causal link to ED in this class of organisms. We have seen (data not shown) that although Vz postpones molt and BPA induces it, both compounds similarly seem to precipitate both sets of exposed isopods to premolt stage. Nevertheless, whilst for BPA exposed isopods the period of time they undergo premolt is normal and ecdysis happens, for Vz the hyperecdysonism does not allow for the necessary exuviation factor to be released. This prolongs the premolt stage, animals show delayed or incomplete molts, and death surmounts at higher toxicant concentrations. But despite the different interference of the two test compounds with molting, here we show that they both caused significant decreases in overall growth, for adult, juvenile, female and male isopods. Actually, there are still uncertainties regarding the sensitivity of different genders, developmental stages, and critical periods of endocrine function in invertebrates exposed to EDCs (Rodriguez et al., 2007). The impacts of EDCs may differ according to the specific life stage at which exposure occurs (e.g., embryolarval stages, gonadal development, etc.) (deFur et al., 1999).
According to Lawlor (1976), in the woodlouse *Armadillidium vulgare*, the total energy allocated to growth plus reproduction among reproductive females is equivalent to the total energy devoted to growth in non-reproductive females or males. This will result in reduced growth rate of reproductive females when included in tests and may lead to extra variability. For this reason, studies concerning toxicant effects on crustaceans should question the use of a mixed set of genders in experiments, regardless of ensuring the withdrawal of visible gravid woodlice from data sets, since these females are able to store sperm and later reproductive processes could be occurring, even if not noticed. Additionally, females not only grow more than males (Vink and Kurniawati, 1996) but also may

have differential responses to the stressor (Weis et al., 1992). In the present study, juvenile virginity was fully assured and separate gender sets used. In the case of tests with adults only males were used. Moreover, animal's uniform age and molt stage was also taken into account, since crustaceans are more susceptible to environmental stresses during molting (Weis et al., 1992)

We investigated the effects of exposure to these two test chemicals on males and females independently. The results show that female isopods are more sensitive than males to BPA exposure (female LOEC at 10 mg BPA/kg soil and male LOEC at 25 mg BPA/kg soil) as evidenced by the reduced weight gain.

Earlier results from Lemos et al. (2009a) with adult male *P. scaber* showed a LC_{50} (95% CI) of 910 (163–1,658) mg/kg soil for BPA and of 298 (150–447) mg a.i./kg soil for Vz after ten weeks of exposure. In the present study, the LC_{50} for the effects on juvenile isopods were higher than 300 mg/kg soil for BPA and 100 mg a.i./kg soil for Vz, even after 16 weeks. Therefore, the lethality of the compounds to the juvenile isopods proved to be in the same order magnitude as for the adults. Nevertheless, juvenile testing was introduced into the experiments in order to assess if this life stage of *P. scaber* is in fact more susceptible in other terms but mortality. The young isopods seemed to respond with reduced growth at lower concentrations (LOECs of 10 mg BPA/kg soil and 100 mg a.i. Vz/kg soil) compared to adults (LOECs of 1 000 mg BPA/kg soil and 300 mg a.i. Vz/kg soil). The increased sensitivity of the juvenile life stage may be either due to easier absorption of the toxicant through their relatively larger body surface/volume ratio and thin cuticle, or due to their lower capacity to metabolize the contaminants (Fischer et al., 1997; Lemos et al., 2009b). Therefore, chronic toxicity assessment ought to be adapted to the lifespan of the woodlouse, from manca to fully reproductive adult as proposed by Samsøe-Petersen (1990).

Although females grew significantly less when exposed to BPA, they did not show a regular concentration-related response to the toxicant. Female-biased sex ratios were reported for juvenile *P. scaber* exposed to this xenoestrogen (Lemos et al., 2009a). The erratic female response to BPA found here may be due to this phenomenon of feminization and the high variability of response that these females show since they might be either phenotypic/genotypic: female/female or female/male.

Juvenile male woodlice grew less than the control from 25 mg BPA/kg soil onwards. The quotient of the LC_{50} value and the geometric mean of the NOEC and the LOEC (LeBlanc, 2004) provides us with an acute:chronic ratio (ACR: >300/$\sqrt{10 \times 25}$) of >19 for BPA exposure. Mu et al. (2005) found an ACR value of 12 for *Daphnia magna* exposed to BPA. An ACR value of >10 is typically indicative of chronic toxicity that is elicited by a mechanism distinct from that responsible for acute toxicity. Also, the growth per molt was significantly lower for isopods exposed to 10 and 1 000 mg/kg BPA (fig. 3.2). Furthermore, we have seen effects on sex-ratios and hormone titres (Lemos et al., 2009a), reproductive endpoints (Lemos et al., 2009b) and protein expression (Lemos et al., 2009c) of *P. scaber* exposed to the same BPA concentrations.

Fukuhori et al. (2005) suggested that the adverse effects seen at high BPA concentrations are the result of systemic toxicity and may not be due to the estrogenic function of the compound. Hence the effects seen at lower concentrations might be from a different origin from those effects seen at higher concentrations. Reports of non-monotonic dose-response curves have accounted as a major challenge in ED risk assessment (Kaiser, 2000; Lemos et al., 2009a). These usually include significant responses to low-doses exposure, while higher doses do not elicit any measurable effects. The classical dose-response paradigm of “the dose makes the poison” is even more complex when similar effects in highest doses are also found (U-shaped dose-response). The possible confounding effects of general toxicity and those that arise from endocrine disruption make these results difficult to interpreter. Hence, it is crucial, at least in a earlier stage, to stretch the range of concentrations tested and to distinguish the maximum tolerated concentration for which effects are not directly associated with lethality (Hutchinson et al., 2009).

3.5. Conclusions

Regardless of the previous reported delayed molting caused by the fungicide Vz and induced molting as result of BPA exposure, both EDCs have elicited overall developmental toxicity in terrestrial isopods. Results show that females are the most sensitive gender to BPA exposure. Juveniles, although having LC_{50} values close to the ones for adults, revealed an increased sensitivity to the toxicants when focusing on chronic effects. Therefore, based in our results, we recommend full life cycle tests in order to provide valuable information regarding differential dose-responses, effects and threshold values that can impair population dynamics by affecting isopod development.

ED low-dose responses were detected for BPA exposure and may have distinct causes from the effects seen at higher doses. Non-monotonic dose-response curves make low-dose effects of endocrine disruptors impossible to predict from high-dose studies. Therefore, extra caution should be paid when defining the concentration ranges to be tested to assess the effects of hormonally active substances and its data interpretation.

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Tables

Table 3.1 - Physico-chemical and mineralogical characterization of the agricultural soil from the lower Mondego valley used in the experiments on the toxicity of Vinclozolin and Bisphenol A to *Porcellio scaber*.

Parameter	value
pH (H_20)	7.48
pH (KCl)	7.31
P (P_20_5)	152 mg/kg
K (K_2O)	180 mg/kg
Na	13 mg/kg
Ca	1478 mg/kg
Mg	53 mg/kg
Fe	16960 mg/kg
Zn	96 mg/kg
Mn	267 mg/kg
Cu	12 mg/kg
Cd	<2.8 mg/kg
Cr	11 mg/kg
Pb	61 mg/kg
Co	<15 mg/kg
Ni	<14 mg/kg
N total	0.06 %
OM	2.4 %
Clay	4.22 %
Silt	7.00 %
Sand	88.77 %
Density	2.4 g/cm^3
WHCmax	70%

Figures

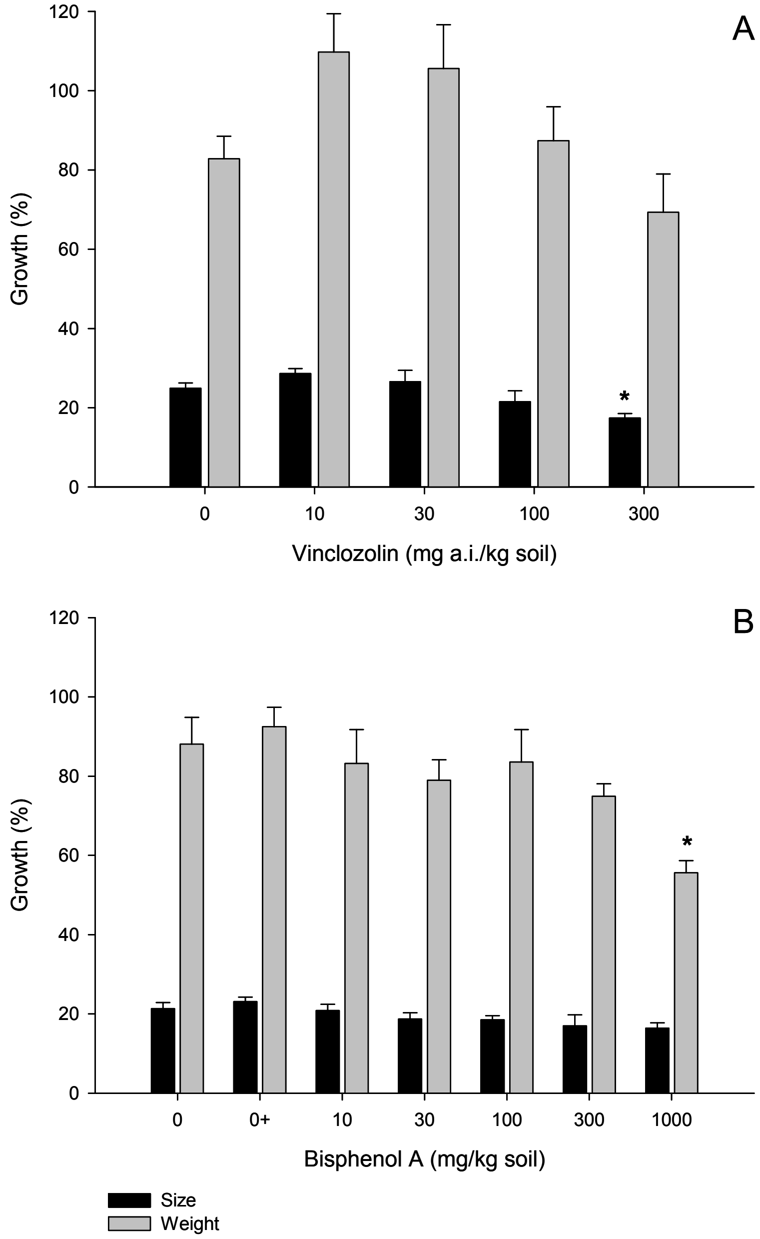

Figure 3.1 - Adult growth of *Porcellio scaber* after 10 week exposure to soil treated with A) Vinclozolin and B) Bisphenol A. Black bars represent size gain (final CW / initial CW) and grey bars represent mass gain (final wt / initial wt). An asterisk indicates a significant difference from the control at $P \leq 0.05$ (ANOVA, Dunnett's test).

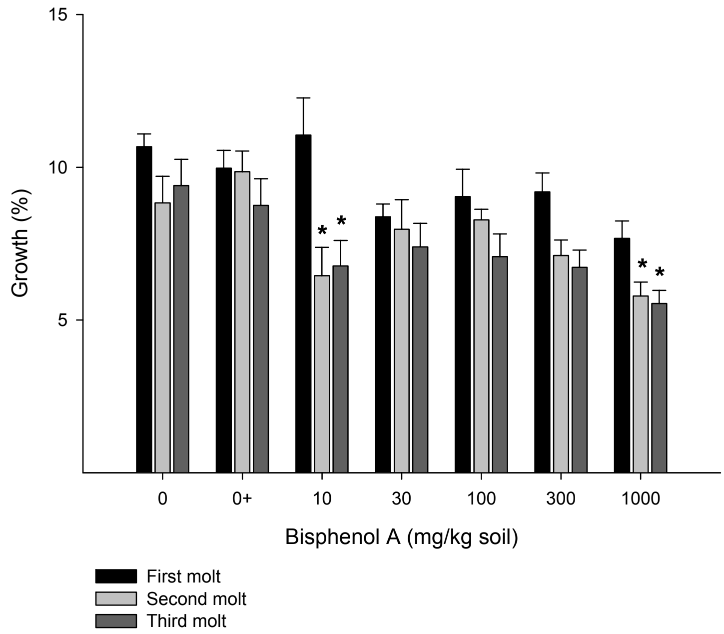

Figure 3.2 - Size gain over three consecutive molts of *Porcellio scaber* after exposure to Bisphenol A contaminated soil. Results are shown as the quotient of the width of the middle segment after and before molt. An asterisk indicates a significant difference from the solvent control at $P \leq 0.05$ (ANOVA, Dunnett's test).

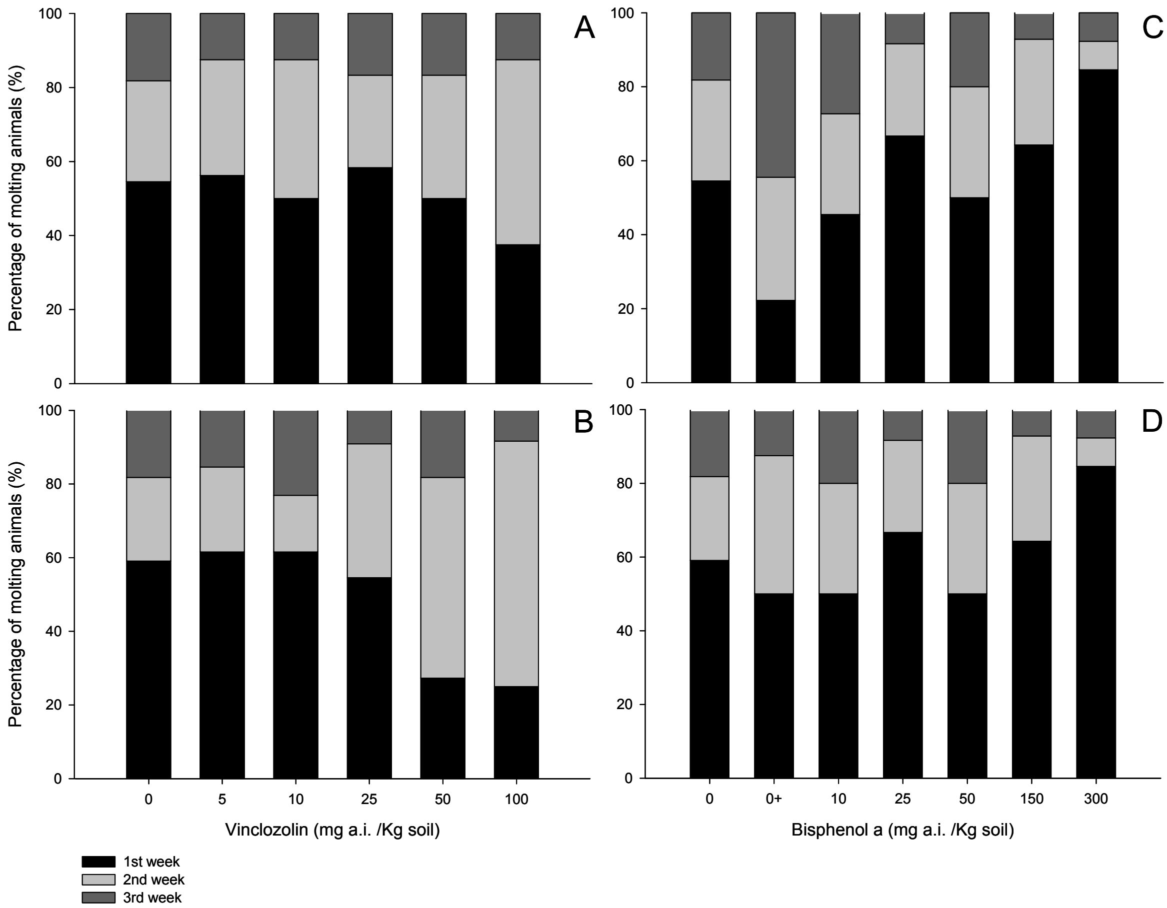

Figure 3.3 - Distribution of first molt per week of juvenile *Porcellio scaber* exposed to soil contaminated with Vinclozolin (left) and Bisphenol A (right); A) and C) males; B) and D) females. Data are presented as percentage of animals that molted during each period.

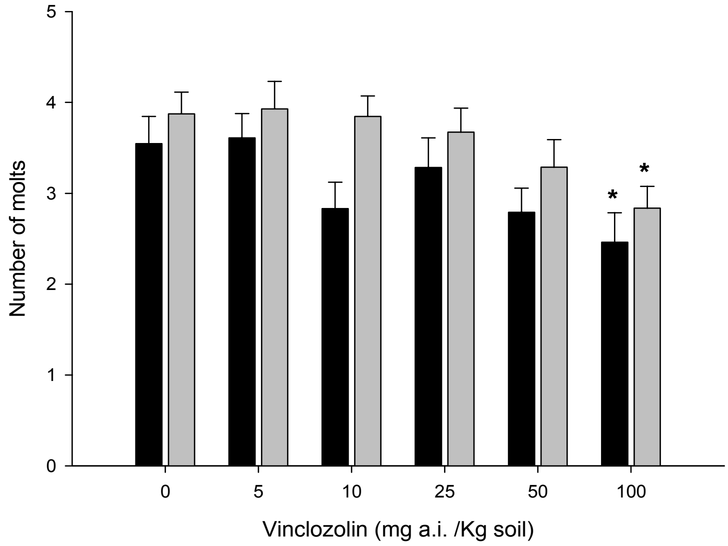

Figure 3.4 - Number of molts of juvenile *Porcellio scaber* over a period of 16 weeks of exposure to soil treated with Vinclozolin. Black represents males, grey females. An asterisk indicates a significant difference from the control at P ≤ 0.05 (ANOVA, Dunnett's test).

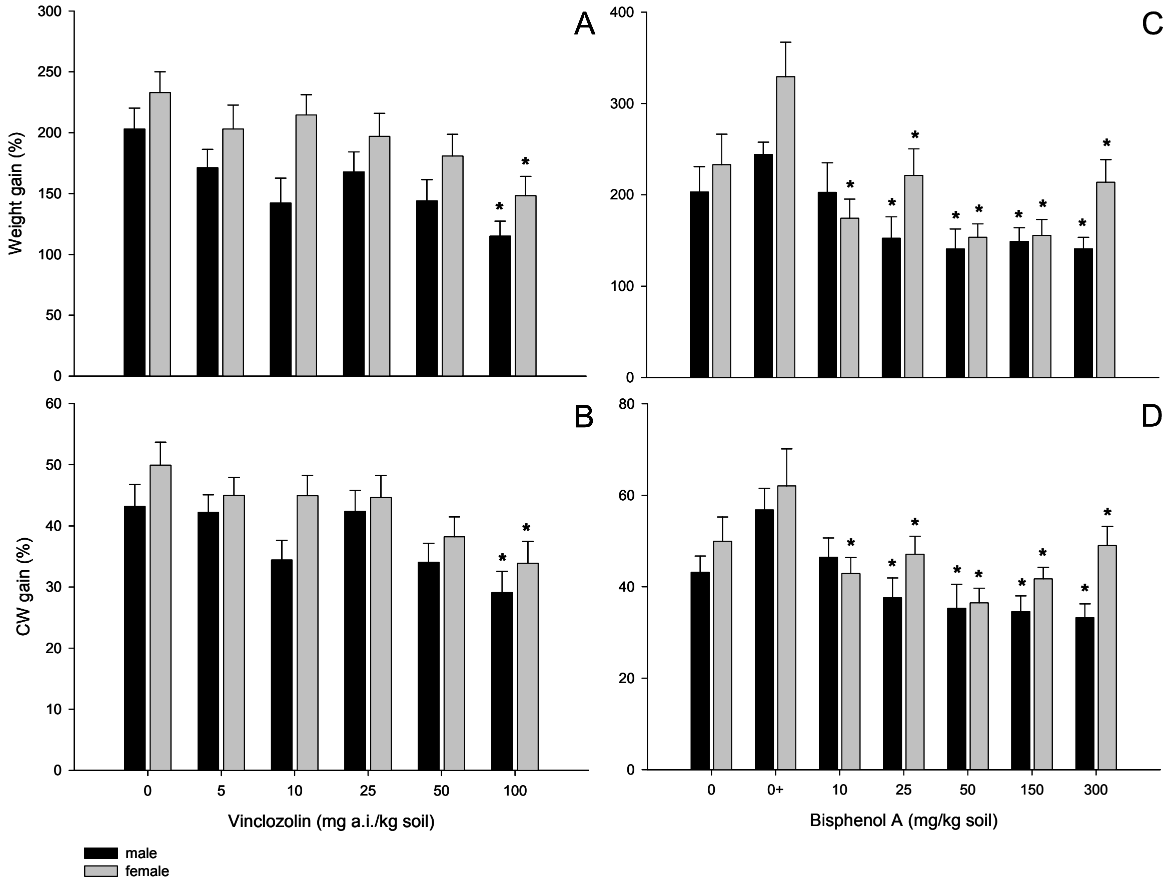

Figure 3.5 - Growth of juveniles *Porcellio scaber* exposed for 16 weeks to soil treated with Vinclozolin (left) or Bisphenol A (right). Black bars represent males and grey bars females. A) and C) mass gain (final wt / initial wt); B) and D) size gain (final CW / initial CW). An asterisk indicates a significant difference from the control at P ≤ 0.05 (ANOVA, Dunnett's test).

Chapter 4

Reproductive toxicity of the endocrine disrupters, bisphenol A and vinclozolin, in the terrestrial isopod *Porcellio scaber*

4. REPRODUCTIVE TOXICITY OF THE ENDOCRINE DISRUPTERS, BISPHENOL A AND VINCLOZOLIN, IN THE TERRESTRIAL ISOPOD *PORCELLIO SCABER*

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Abstract

Background, aim, and scope Endocrine Disruptor Compounds (EDCs) have been largely studied concerning their effects on vertebrates. Nevertheless, invertebrates as targets for these chemicals have been neglected and only few studies are available. Specifically on terrestrial isopods, data concerning the effects of EDCs is limited, although these have a key role in the organic matter decomposition in soils and therefore in soil ecosystem processes. Influences of EDCs on the reproduction systems of these organisms, with consequences at the population level, are expected but have not been confirmed until now.

Materials and methods *Porcellio scaber* adult organisms were exposed to increasing concentrations of Vinclozolin (Vz) and Bisphenol A (BPA) for 56 days. Woodlice were mated and the percentage of females that successfully reached pregnancy, the time until pregnancy, duration of pregnancy and the percentage of abortions were recorded. Juvenile number born per female, juvenile weight, percentage of juveniles alive, juvenile weight increase after two months and reproductive allocation (RA) were also registered.

Results The average time at which female showed the first signs of pregnancy was lower for isopods exposed to 1000 mg a.i. Vz/kg dry soil but not for animals exposed to BPA. The average pregnancy duration also decreased with increasing Vz concentrations but was not affected by BPA exposure. Nevertheless, increasing concentrations of BPA induced a decrease on the success to achieve pregnancy and an increase of miscarriages at 10 and 1000 mg/kg dry soil. Only 40% of the females became pregnant at the highest Vz concentration and of these none were able to deliver mancae. The average number of mancae delivered per female decreased with increasing concentrations of Vz but individual manca weight increased. BPA seemed to have no effect on these parameters. Juvenile survival was strongly affected by Vz, but not by BPA. On the other hand, reproductive effort decreased with increasing BPA and Vz concentrations.

Discussion Isopods exposed to Vz and BPA decreased their reproductive allocation, which is usually a consequence of a trade-off favouring survival rather than growth and/or reproduction. The reduced fecundity and highly increased abortion rate found for both Vz and BPA may be related to the hormonal de-regulation previously detected at the same concentrations. The high 20-hydroxyecdysone (20E) levels have been proven to interfere with the molt cycle, which is inextricably linked with the female reproductive cycle. This hyperecdysonism might also be interfering with vitellogenin, which is a precursor of egg yolk proteins and therefore contributing to the disruption of reproductive processes.

Conclusions The methodologies presented here are suitable for toxicity assessment of EDC-exposed isopods. Furthermore, the results of the present study confirm that Vz and BPA elicit toxicity to several parameters related to terrestrial isopod reproduction. Vz reduced pregnancy duration, increased the abortion percentage, decreased the number of pregnancies, and decreased of number of juveniles per female while BPA increased abortions at the lowest and highest test concentrations.

Recommendations and Perspectives The reproductive endpoints presented in here are indicative of the possible impact that this type of compounds might have on isopod population dynamics, which may eventually lead to population decline. Moreover, the influence of this class of toxicants on the reproductive capacity of terrestrial isopods should be further investigated, namely concerning transgenerational effects.

Keywords Endocrine disruptor compound • Reproductive toxicity • Bisphenol A • Endocrine Disruption • Ecotoxicological testing • *Porcellio scaber* • Terrestrial isopods • Vinclozolin • Vitellogenin • Reproductive allocation • Ecdysteroids

4.1 Background, aim, and scope

There are increasing evidences that environmental contaminants are impacting wildlife populations by interfering with some aspects of endocrine-mediated processes, causing impairments of developmental, reproductive and other hormonally mediated processes (Rodriguez et al., 2007). These chemicals, termed endocrine disruptor compounds (EDCs), are either natural, synthetic and industrial chemicals or by-products suspected to alter the functions of the endocrine system, and consequently causing adverse health effects in an intact organism, or its' offspring, or (sub)population (European Commission, 2007). While for vertebrates significant efforts have been made to study the effects of EDCs, to date there are very few studies on the effects of these compounds on invertebrates, although they constitute the vast majority of organisms of the animal kingdom (deFur et al., 1999). Chemicals may disrupt molting processes by interfering with the production or function of molting hormones (ecdysteroids, arthropods' major signalling molecules) (LeBlanc, 2007). Although molting is a mechanism of high relevance to arthropods it has no relevance to vertebrates and provides a way by which contaminants may impact crustacean species while not affecting vertebrates. Thus, when considering the endocrine toxicity of environmental chemicals to invertebrates, it is critical that the susceptibility of invertebrate species is directly evaluated rather than inferred from other groups (deFur et al., 1999).

Isopods are widespread saprophytic organisms that carry a key role in the organic matter decomposition in soils. Laboratory testing using terrestrial isopods has been recommended for assessing the ecotoxicological effects of chemicals (Drobne and Hopkin, 1994). Moreover, these organisms combine the features of continuous growth through a molting regime, sexual reproduction, relative good knowledge of endocrinology and a terrestrial mode of life, making them suitable candidates for sentinel species for these types of pollutants in terrestrial environments (Lemos et al., 2009a).

In terrestrial isopods, the female reproductive cycle (ovarian maturation and embryogenesis) is an overlapping/synchronous event along with the molt cycle

(Subramoniam, 2000). Ecdysteroids, as chief hormonal factors, have been demonstrated to be necessary for vitellogenin (vtg) synthesis (Vafopoulou and Steel, 1995), the precursor of egg yolk proteins. The coordinated control of molting and reproduction is achieved by this ecdysteroid/vtg inter-relationship mechanism with each reproductive cycle having the length of one molt cycle, and with the vitellogenic processes being completed during an extended intermolt period, with spawning occurring after ecdysis (Subramoniam, 2000).

In this study, two compounds with proven endocrine activity were investigated. Vinclozolin (Vz) is a fungicide widely used in agriculture for the production of lettuce, raspberries, beans, onions, vine and others. It is a proven endocrine disrupter causing anti-androgenic effects due to two of its metabolites, which are able to bind to the androgen receptor (Anway et al., 2005). Bisphenol A (BPA) is an industrial compound that has generated concerns due to its high production and widespread use in many consumer products and its proven estrogenicity (Okada et al., 2008). Many cases of potential adverse effects of BPA on humans as well as wildlife have been reported (Crain et al., 2007 for a review).

The aim of this study was to determine the effects of these two EDCs on several reproductive parameters in the terrestrial isopod *Porcellio scaber*, when exposed to contaminated soil. The investigations presented are a part of a broader attempt to improve the knowledge of the effects of EDCs on edaphic arthropods.

4.2. Materials and Methods

4.2.1 Test organism and culture procedures

The isopods, *Porcellio scaber* Latreille (1804), were obtained from a laboratory culture initiated 8 years earlier with animals collected from horse manure in central Portugal. Animals were cultured in plastic boxes with a layer of sterile sand, fed *ad libitum* with oven-dried alder leaves (*Alnus glutinosa*) and maintained at 21º±1ºC, with 16:8 (light:dark) photoperiod and 100% relative air humidity.

Gravid females, with fully-formed marsupium, were placed in a secondary culture until release of mancae. Isopods born within the same day were used to start a synchronized culture. Once they attained a weight of 4-5 mg they were reared individually in polyethylene terephthalate (PET) boxes (Ø 60 x 30 mm) perforated on the sides to ensure ventilation and under the same conditions described above.

4.2.2. Chemicals and preparation of test soils

An agricultural natural soil kept in fallow for at least five years was collected from the lower Mondego valley (Portugal). The soil was oven dried at 60ºC for 48 hours. Bisphenol A (BPA, Merck Schuchardt, Germany, purity >99%) was dissolved in methanol and mixed in with the soil at 10, 30, 100, 300 and 1000 mg/kg dry soil. Subsequently, methanol was allowed to evaporate under a fume hood for 12 hours and moisture was adjusted to 20% (v/w) with distilled water. A solvent control was prepared in the same way, using the same volume of methanol without BPA (represented as 0^{+}). A control was prepared with water added to dry soil.

Vinclozolin (Ronilan® 50% active ingredient; BASF AG, Germany) was mixed with soil at concentrations of 10, 30, 100, 300 and 1000 mg a.i./kg dry soil. Afterwards, the soil was moistened to 20% (v/w) with distilled water. A control was prepared with water added to dry soil.

Chemical analysis of Vz and BPA treatments concentration were made and were within ± 5% of the nominal concentrations. The results are presented in terms of the nominal values.

4.2.3 Reproduction test with *Porcellio scaber*

Male and female isopods weighing 20±1 mg were taken from the synchronized individual culture in order to ensure virginity. Only animals in the intermolt stage were used, ensuring that all organisms were at the same vulnerability stage.

Woodlice were randomly paired, one male and one female per box (Ø 100 mm x 50 mm PET boxes), using 10 boxes per concentration. Each box contained 60 g of agricultural spiked soil and four Ø 10 mm alder leaf discs providing same shelter area for all the animals. Food was added weekly in order to maintain it in excess and to have an equal number of discs in all boxes. Experiments were performed under the same conditions as described for the *P. scaber* cultures.

Female reproductive cycle was followed for 56 days after mating and the following parameters were recorded: time to reach pregnancy; pregnancy duration as time between the first observation of gravidity to first observation of released mancae; percentage of females that successfully reached pregnancy within the 56-day test period; percentage of abortions as the fraction of females that successfully reached pregnancy but were unable to carry it to the end; number of juveniles per female; juvenile weight calculated as the difference in female weight before and after parturition divided by the number of mancae; percentage of live juvenile after two months; juvenile weight increase after two months; and reproductive allocation (RA) as the quotient of total juvenile fresh weight and female fresh weight after parturition.

4.2.4 Statistical analysis

All data were checked for normality and homoscedascity. One way analysis of variance (ANOVA) with Dunnett's multiple comparison of means were employed to determine differences relatively to control treatment. Where applicable, results are presented as mean ±SE. For all statistical tests the significance level was set at $P \leq 0.05$. All calculations were performed with SigmaStat software package (Systat Software Inc., 2006).

4.3. Results

4.3.1 Lethality of Vinclozolin and Bisphenol A

The eight weeks LC_{50} (95% CI) for Vz was 824 (512–1135) mg a.i./kg dry soil. Exposure to 1000 mg BPA/kg dry soil led to 30% mortality, thus to a LC_{50} value higher than 1,000 mg/kg dry soil. Since the concentration range of Vz include the LC_{50}, the higher mortality of adults and juveniles at higher Vz concentrations provoked a low number of replicates (e.g. at the highest concentration, the reproductive success was zero). This contributed to the impossibility of statistically distinguishing significant differences in several parameters tested, despite the visibility of trends, as in the case of the time to reach pregnancy, juvenile survival and growth and RA (figures 4.1, 4.4A and 4.5A). Moreover, in cases where no data is available due to higher mortality, the concentration is omitted from the figures.

4.3.2 Time to reach pregnancy and pregnancy duration

In the controls, the average time after mating at which female isopods showed the first signs of pregnancy was of 23±3 days. This interval was lower for isopods exposed to 1000 mg Vz/kg dry soil (11±1 days), but this difference was not statistically significant (ANOVA, Dunnett's test, $F_{5.34}$= 1.702; P= 0.161) (fig. 4.1). This reduced time to achieve pregnancy in the highest concentration is probably due to the fact that only earlier pregnancies were considered while later pregnancies were not found due the mortality in later periods**.** Average pregnancy duration was of 30±1 days for the control and decreased with increasing Vz concentrations. This decrease was statistically significant for isopods exposed to 300 mg Vz/kg dry soil compared to control (ANOVA, Dunnett's test, $F_{4.23}$= 3.226, P= 0.031). At 1000 mg a.i./kg none of the animals were able to complete pregnancy (fig. 4.1).

BPA did not affect the time to reach pregnancy, which was 21±2 days for the solvent controls, nor the pregnancy cycle duration, of 29±1 days for solvent control (ANOVA, Dunnett's test, $F_{5.36}$= 0.141, P= 0.982 and $F_{5.34}$= 0.845, P= 0.528, respectively).

4.3.3 Occurrence of pregnancies and abortions

All control and solvent control females successfully attained gravidity within the test period. Only 40% of the females became pregnant at the highest Vz concentration (fig. 4.2A). The percentage of abortions increased with Vz concentration in a dose-dependent way with none of animals exposed to 1000 mg Vz/kg dry soil being able to deliver mancae.

The success to achieve pregnancy slightly decreased with increasing BPA concentrations, with 30% of the females unable to become pregnant at 1000 mg BPA/kg dry soil. In the solvent control, all females carried the pregnancy until the end, but at 10 and 1000 mg BPA/kg dry soil there were 20% miscarriages (fig. 4.2B).

4.3.4 Number of juveniles per female and their individual weight

The average number of mancae delivered per female was 19±2 in the controls and decreased with increasing concentrations (fig. 4.3), with a LOEC of 100 mg Vz/kg dry soil (13±1 mancae for females exposed to 100 and 300 mg a.i./kg dry soil) (ANOVA, Dunnett's test, $F_{4.29}$= 3.242, P= 0.026). Associated with the decreasing number of mancae per female, the individual manca weight increased with increasing Vz concentration (19.7 and 22.0% heavier juveniles at 100 and 300 mg Vz/kg dry soil, respectively), although no statistically significant differences are found (ANOVA, Dunnett's test, $F_{4.29}$= 1.080, P= 0.385).

The number of mancae born was lower in the solvent control compared with the water control (t-test, DF= 14, t= 2.443, P= 0.028) and was not affected by BPA treatment (ANOVA, Dunnett's test, $F_{5.35}$= 0.316, P=0.900). Weight of individual newborns did not differ between treatments and the solvent control (ANOVA, Dunnett's test, $F_{5.33}$= 0.439, P= 0.818).

4.3.5 Surviving juveniles and their growth after 8 weeks

After 8 weeks exposure to Vz, 71% of the juveniles survived in the control and only 53% and 25% at 100 and 300 mg a.i./kg dry soil, respectively (fig. 4.4A), but without statistical significance (ANOVA, Dunnett's test, $F_{4.20}$= 1.128, P= 0.371). After that period, control isopods weighed 3.19 ±0.29 mg with a slightly higher weight gain at lower concentrations and a highly reduced juvenile growth at higher Vz concentrations (1.77 ±0.30 mg and 1.30 ±0.91 mg for 300 and 1000 mg Vz/kg dry soil, respectively) although this effect was not significant (ANOVA, Dunnett's test, $F_{4.20}$= 1.66, P= 0.211).

After 8 weeks exposure to BPA, juvenile survival and growth showed no clear dose-response relationship (fig. 4.4B). Significant effects were found on survival (ANOVA, Dunnett's test, $F_{5.31}$= 3.270, P= 0.017), but the post hoc test was not able to distinguish between treatments. Survival was increased at the lowest concentration. Juvenile growth was stimulated (55%) at 10 mg BPA/kg dry soil but no significant differences between treatments and solvent control were found (ANOVA, Dunnett's test, $F_{5.31}$= 1.317, P= 0.284).

4.3.6 Reproductive allocation (RA)

Reproductive effort, measured as reproductive allocation (RA), tended to decrease with increasing Vz concentrations (23.5% lower at 300 mg a.i./kg dry soil), but effects were not significant (ANOVA, Dunnett's test, $F_{4.23}$= 0.707, P= 0.596) (fig. 4.5A).

The reproductive effort decreased with increasing BPA concentrations. At 1000 mg BPA/kg dry soil, 41.6% less resources were allocated to reproduction than in the solvent control (ANOVA, Dunnett's test, $F_{5.33}$= 2.621, P= 0.042) (fig. 4.5B). An apparent low concentration effect was also seen with 20.4% less RA at 10 mg BPA/kg dry soil.

4.4. Discussion

In the controls, all females became pregnant and delivered mancae. These values are much higher than the few other reports with other isopod species. Van Brummelen et al. (1996) reported a 57% failure of gravidity in *Oniscus asellus*, Faber and Heijmans (1995) reported a 49% failure in *Trachelipus rathkei*, and Hornung and Warburg (1994) reported a 47% failure in *P. ficulneus*. These studies demonstrate the suitability of the experimental design, methodologies and species used in here.

The vinclozolin applied to the soil was in the form of the commercially available formulation, Ronilan® (BASF, AG), containing 50% active substance, and bisphenol A as a self-made methanol solution which was evaporated previously to the exposure of the animal. Although it cannot be completely excluded that the higher toxicity of Vz is due to unknown compounds included in the formulation of Ronilan®, we could not find any information that supports this as a confounding factor.

Living organisms allocate their available resources to growth, reproduction, basal metabolism, and/or to improve survival, in a combination designed to maximize fitness (Sibly and Calow, 1986). The physiological cost of combating stress caused by exposure to contaminants may give rise to trade-offs in resource allocation as the animal tries to optimize its fitness under the altered environmental conditions, increasing energy expenditure for the basal metabolism in order to cope with the stress. Ultimately this may lead to the reduction of growth and reproduction.

It has been shown that the terrestrial isopods *P. scaber* and *O. asellus* from metal polluted sites comprise high reproductive investment suggesting that they were able to redirect resources from other functions, like growth, to meet the physiological costs of metal detoxification (Jones and Hopkin, 1996). Donker et al. (1993) found that exposure to metals caused increased mortality and slower growth rates in isopod populations, and that this has selected for early reproduction and greater RA. Faber and Heijmans (1995) also noted an increase in RA for *T. rathkei* exposed to phenanthrene, when these isopods had a history of PAH contamination.

A similar increase of RA was not seen for either Vz or BPA. *P. scaber* showed a trend to reduce the reproductive effort at higher concentrations. Because growth, survivorship and reproduction are targets of selection, life-history theory predicts that habitat disturbances which result in higher adult mortality rates can select for early maturation and increased reproductive effort (Sibly and Calow, 1989). Accordingly, nearly all studies on the breeding phenology of isopods, which have been done using populations with a history of contaminant exposure (e.g. Donker et al., 1993; Jones and Hopkin, 1996), have shown evidence of early reproduction and increased RA. This could be due to changes as result of evolutionary pressures by means of environmental stress drivers (Farkas et al., 1996; Donker et al., 1993). In the present study, and because the isopods had no prior toxicant exposure history, the mentioned opposite responses in reproductive effort might thus be due to the lack of such an evolutionary pressure with results showing a trade-off which favours survival (i.e. more energy allocated to detoxification) rather than growth and/or reproduction.

Of main importance in the crustacean female reproductive cycle is the inextricable link with the molt cycle. The continuous body growth and periodic molting is not deterred by reproduction, except when the eggs are bred in the pleopods, which prevents the onset of the next molting (Subramoniam, 2000). For this reason, the integration of molting with reproduction is a physiological need in female crustaceans. Additionally, it is known that 20-hydroxyecdysone (20E) levels regulate the molting process (Horn et al., 1966). Investigations where crustaceans were injected with 20E showed a precipitation of premolt changes in the cuticle leading to precocious ecdysis (Gunamalai et al., 2004). This may cause the attached embryos to quicken the hatching process and the brood may be lost during the ensuing molt (Gunamalai et al., 2004).

In a previous study (Lemos et al., 2009a), increased levels of 20E were found in male isopods exposed to 10 and 1000 mg BPA/kg dry soil and also with increasing exposure concentrations of Vz. This effect is in agreement with the reduced fecundity and highly increased abortion rate found for both Vz and BPA in this study, at the same exposure concentrations. This may well be the result of the hormonal de-regulation induced by these toxicants.

The reported hyperecdysonism might also be interfering with vtg, a key protein of extreme importance in reproduction, which is a precursor of the egg yolk proteins (Gohar and Souty, 1984). Increased 20E titres have been correlated with increased yolk protein synthesis and uptake in developing oocytes (Gohar and Souty, 1984), in this way contributing to disruption of reproductive processes. Vtg levels may increase when animals are exposed to contaminants with feminising effects (Marin and Matozzo, 2004). Induction of vtg production following BPA exposure in fully organized individuals has been reported for fish, amphibians and invertebrates (for a review see Crain et al., 2007). The stimulatory effect of 20E on vitellogenesis is known to increase the amount of accumulated vitellin in oocytes to be utilized as a source of nutrients during embryonic development (Okumura et al., 1992). The higher amount of available energy reserves may lead to bigger juveniles even when brooding periods are shorter, as in the case of isopods exposed to Vz concentrations of 100 and 300 mg/kg dry soil. Another potential reproductive trade-off for stressed females can be the increased number of atretic eggs (Hornung and Warburg, 1994), a strategy resulting in the reuptake of energy for investment in the remaining eggs and juveniles in the marsupium. Added to this, the fact that brood-pouch mortality might be favouring survival of more developed juveniles, it may justify both the reduced number of offspring and their increased size. Also noteworthy is that since the isopod marsupium provides only a limited area for egg attachment (Lardies et al., 2004), abnormal egg growth may lead to overcrowding and consequent egg loss or reabsorption, resulting in lower mancae numbers (Lardies et al., 2004).

We have shown that vinclozolin induces a decrease of the brood period. A similar observation was seen in female *Armadillidium vulgare* exposed to ants (Castillo and Kight, 2005) and female *P. leavis* under physical stress (Kight and Nevo, 2004), with stressed females releasing the juveniles almost 48 hours earlier than controls. Parental care by female isopods is energetically costly (Lardies et al., 2004). Under stress, this behaviour may represent a trade-off due to the reduction in the amount of metabolic reserves, impairing the successful provision of the juveniles, thereby reducing the length of time a female has to provision for her developing young (Castillo and Kight, 2005). In our study, in the presence of Vz, the isopods released juveniles almost 43 hours earlier at 100 mg a.i./kg dry soil and five days earlier at 300 mg a.i./kg dry soil.

Smaller freshly released mancae of *P. scaber* seem more susceptible to toxicants than more developed juveniles (Fischer et al., 1997). This may be either due to easier absorption of the toxicant through their relatively larger body surface/volume ratio and thin cuticle, or by their lower capacity to metabolize the contaminant. Although being released more developed at higher Vz concentrations, juvenile toxicity due to contamination cannot be excluded. After 2 months, juveniles had grown less at the higher concentrations than at lower concentrations where they were released with lower weights.

Ecdysteroids can bind to vitellin, suggesting that they can be maternally transferred to oocytes (Subramoniam, 2000) and are critical to the normal crustacean embryo development (Mu and LeBlanc, 2002). Therefore, there is no reason to believe that high 20E titres, reported to have negative effects in adults, will not have similar negative effects in the juveniles, once they are maternally transferred.

Ecdysteroids also have been shown to directly or indirectly regulate some aspects of spermatogenesis emphasizing their importance as reproductive hormones (Gohar and Souty, 1984). Additionally, ecdysteroids were also involved in oocyte maturation in the prawn *Palaemon serratus* (Lanot and Cledon, 1989). The first meiotic resumption corresponded to the accumulation of ecdysteroids in oocytes at the premolt stage, and 20E also induced germinal vesicle breakdown *in vitro* (Okumura and Aida, 2000). Therefore, toxicants that interfere with 20E metabolism, as is the case for BPA and Vz (Lemos et al., 2009a), will most certainly affect male and female reproduction. Additionally, Lemos et al. (2009b) found that BPA induced the up-regulation of tubulin in isopods' testes. Such a tubulin up-regulation has been shown to lead to aneuploidies in the sea urchin (George et al., 2008) and consequently may lead to fecundity problems and non-viable progeny.

After BPA exposure, 70% of the females became pregnant at the highest concentration tested. Of them, 29% did not manage to carry their pregnancy to the end. For the lowest BPA concentration, we also observed the same tendency, with 20% of females being unable to attain pregnancy, and of the rest 25% aborted. For Vz, we saw a decreasing number of females successfully attaining pregnancy associated with an increased number of miscarriages along with increasing concentrations of the fungicide. These two factors together considerably reduced the total juvenile output. Fischer et al. (1997) reported a lower reproduction in *P. scaber* exposed to the insecticide dimethoate, which was not due to a decreased number of mancae per female but rather the consequence of a lower proportion of gravid females, suggesting this is the most sensitive reproductive parameter.
The lack of sensitivity of crustacean development and reproduction to estrogens and xeno-estrogens at sublethal concentrations has been reported by several authors (e.g. Bechmann, 1999; Hutchinson et al., 1999). Nevertheless, there are numerous examples of xeno-oestrogen effects in invertebrate reproductive traits. The xeno-estrogens BPA, 4-tert-octylphenol and 4-nonylphenol (NP) caused significant stimulation of embryo production in the freshwater mudsnail *Potamopyrgus antipodarum* (Duft et al., 2003). BPA also stimulated the reproductive output in *Acartia tonsa* (Andersen et al., 1999). Brown et al. (1999) found both a lower survival and higher fertility in the NP exposed amphipod *Corophium volutator*. A complex "superfemale" syndrome, characterized by massive stimulation of oocyte and spawning mass production, was reported in the BPA exposed freshwater ramshorn snail *Marisa cornuarietis* and the marine gastropod *Nucella lapillus* (Oehlmann et al., 2000). A structural resemblance of PAHs to estrogens was suggested by Van Brummelen et al. (1996) as an explanation for the stimulatory effect on overall reproduction (increased proportion of gravid females and induction of egg-laying) in *P. scaber*. Albeit the many reports pointing at the stimulatory effects of xeno-estrogens, here we were unable to detect such effect for *P. scaber* exposed to BPA or to Vz. The limited size of the marsupium (Lardies et al., 2004) may restrict the final number of viable offspring a female can deliver (as explained above), making the number of released mancae

probably not the most suitable parameter to assess an eventual “superfemale” syndrome caused by xeno-estrogens.

Reproduction toxicity should be classified as systemic toxicity and not as ED, unless specific parameters such as hormone levels are affected. If only fecundity is evaluated, more evidence is needed to conclude that the inhibiting effect of a pollutant is due to endocrine disruption (Barata et al., 2004). In the present study, reproductive impairment has been demonstrated for compounds with proven ecdysteroidal activity (Lemos et al., 2009a). Ecdysteroids are, undoubtedly, the chief hormone factors controlling development and reproduction (Gilbert et al., 2002). Nevertheless, alongside with ED, we can not exclude that systemic toxicity, such as lethality on the eggs and mancae, may play a fundamental role in the reproductive impairment.

The concentration of vinclozolin in the soil, after the maximum recommended application rate of Ronilan®, is of 1 mg a.i./kg (assuming that 70 % of the fungicide will reach the surface and is homogeneously distributed over the top 5 cm soil layer and the soil bulk density is 1.4 kg/dm^3) (Lemos et al., 2009a). Also, the only significant route of BPA to the terrestrial environment is through the application of sewage sludge from municipal plants [concentrations of 0.033-36.7 mg/kg (dw)] (Lee and Peart, 2000) in the land to function as soil improver (Furhacker et al., 2000), indicating that contamination of agricultural soils is a real issue. Whilst the results presented in this work are indicative of the chronic toxicity of these compounds, exposure concentrations at which these effects are elicited were well above the ones expected to be found in the environment for Vz whereas some caution should be paid to the ratios at which sludge should be mixed in the land.

4.5 Conclusions

The results of the present study confirm that bisphenol A and vinclozolin elicit overall reproductive toxicity in terrestrial isopods with decreasing reproductive allocation for the exposed females. Vz reduced pregnancy duration, increased the abortion percentage, decreased the number of pregnancies, and decreased the number of juveniles per female while BPA increased abortions at the lowest and highest exposure levels. The already reported 20E level variation (Lemos et al., 2009a) was pointed out as possible cause for embryo development toxicity. This hyperecdysonism, with the consequent 20E interference in vitellogenic and molt cycle processes are also means by which Vz and BPA probably reduce the overall reproductive output.

4.6 Recommendations and perspectives

On the premise that reproductive success is the ultimate vital population parameter (Hutchinson, 2007), the overall fecundity, the reproductive success, and the juvenile output and survival are indicative of the possible impact that this type of compounds might have on isopod population dynamics, which may eventually lead to population decline. Moreover, due to the specific modes of action of EDCs, transgenerational effects should also be addressed in the future.

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Figures

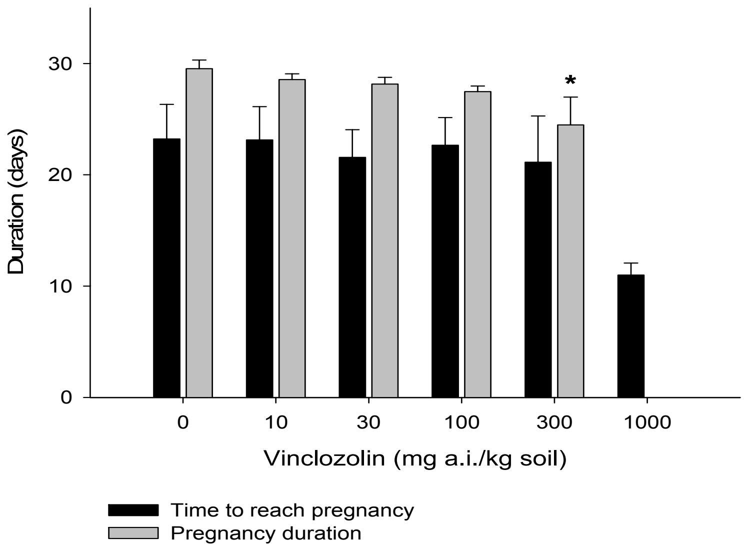

Figure 4.1 - Number of days until first signs of pregnancy were detected (black bars), and number of days between first signs of pregnancy and release of mancae (grey bars) of *Porcellio scaber* exposed to soil treated with Vinclozolin. For the time to reach pregnancy *n* ranges from 6 to 9 in all experimental groups except in the group exposed to 1000 mg a.i./kg of dry soil, where *n* is 4, due to lower number of pregnancies. Vertical error bars represent the standard error of the mean. An asterisk indicates a significant difference from the control at P ≤ 0.05 (ANOVA, Dunnett's test).

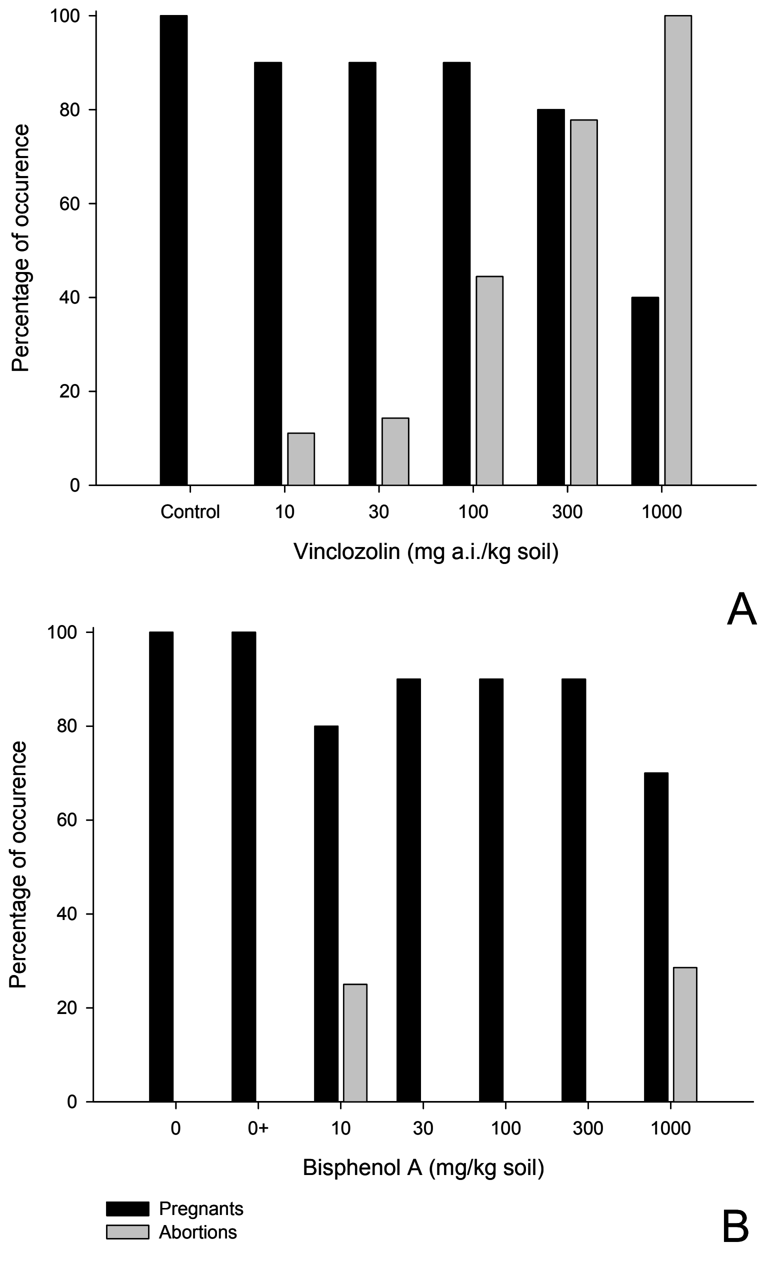

Figure 4.2 - Percentage of successful females achieving pregnancy (black bars) and percentage of female miscarriages (grey bars) of *Porcellio scaber* exposed to soil treated with A) Vinclozolin and B) Bisphenol A.

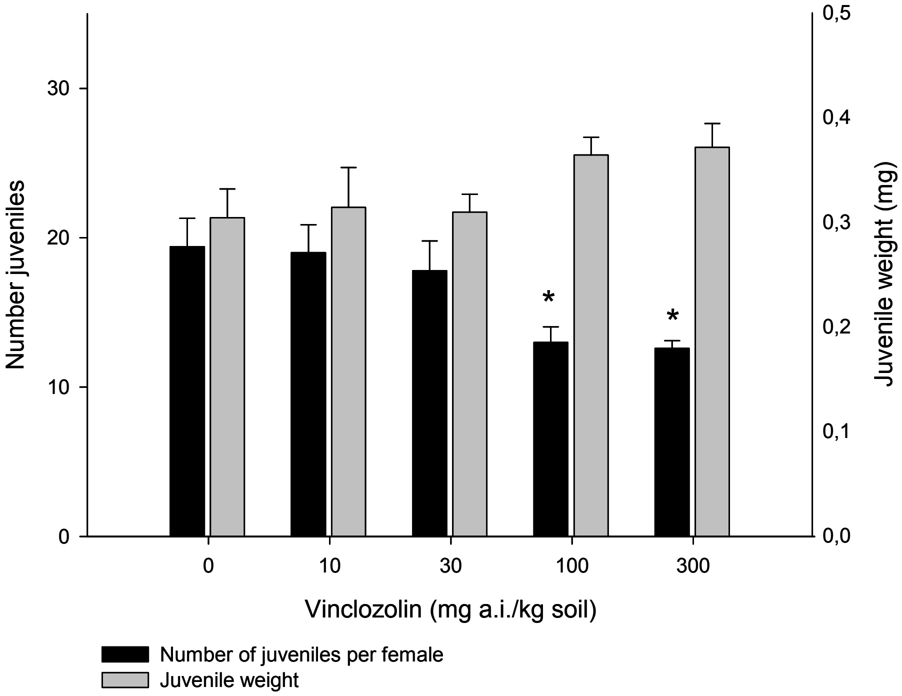

Figure 4.3 - Number of juveniles hatching per each pregnant female (black bars), and individual manca weight (grey bars) of *Porcellio scaber* exposed to soil treated with Vinclozolin. Vertical error bars represent the standard error of the mean. An asterisk indicates a significant difference from the control at P ≤ 0.05 (ANOVA, Dunnett's test).

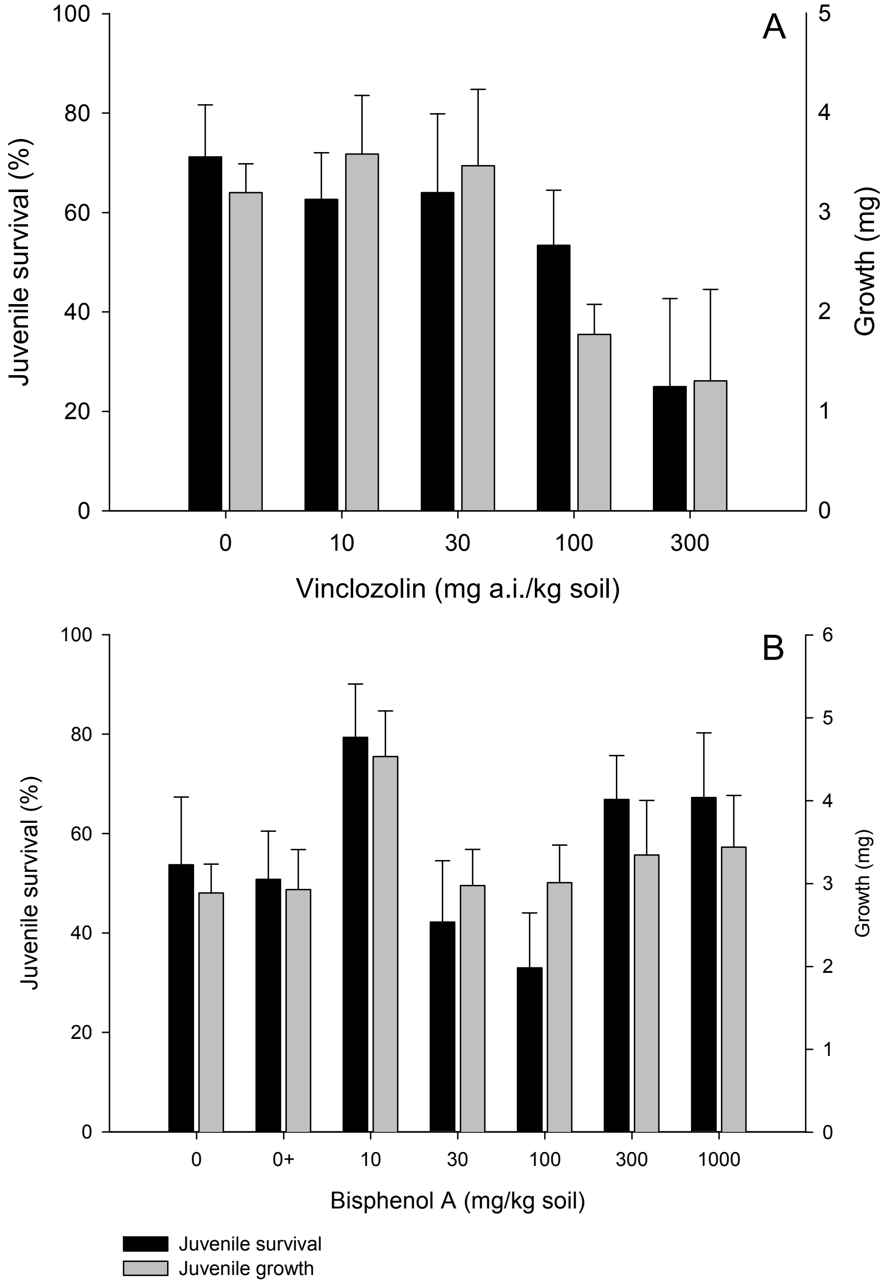

Figure 4.4 - Percentage of surviving juveniles (black bars), and juvenile increase of weight (grey bars) in *Porcellio scaber* after a two months exposure to soil treated with A) Vinclozolin (control group *n* = 8; at 10 mg a.i./kg *n* = 7; at 30 mg a.i./kg *n* = 4; at 100 and 300 mg a.i./kg *n* = 3) and B) Bisphenol A (control group *n* = 8; solvent control *n* = 6; at 10 mg/kg *n* = 4; at 30 mg /kg *n* = 8; at 100 mg /kg *n* = 6; at 300 mg /kg *n* = 8; and at 1000 mg /kg *n* = 4). The decrease of replicates along treatments is due to juvenile mortality. Vertical error bars represent the standard error of the mean.

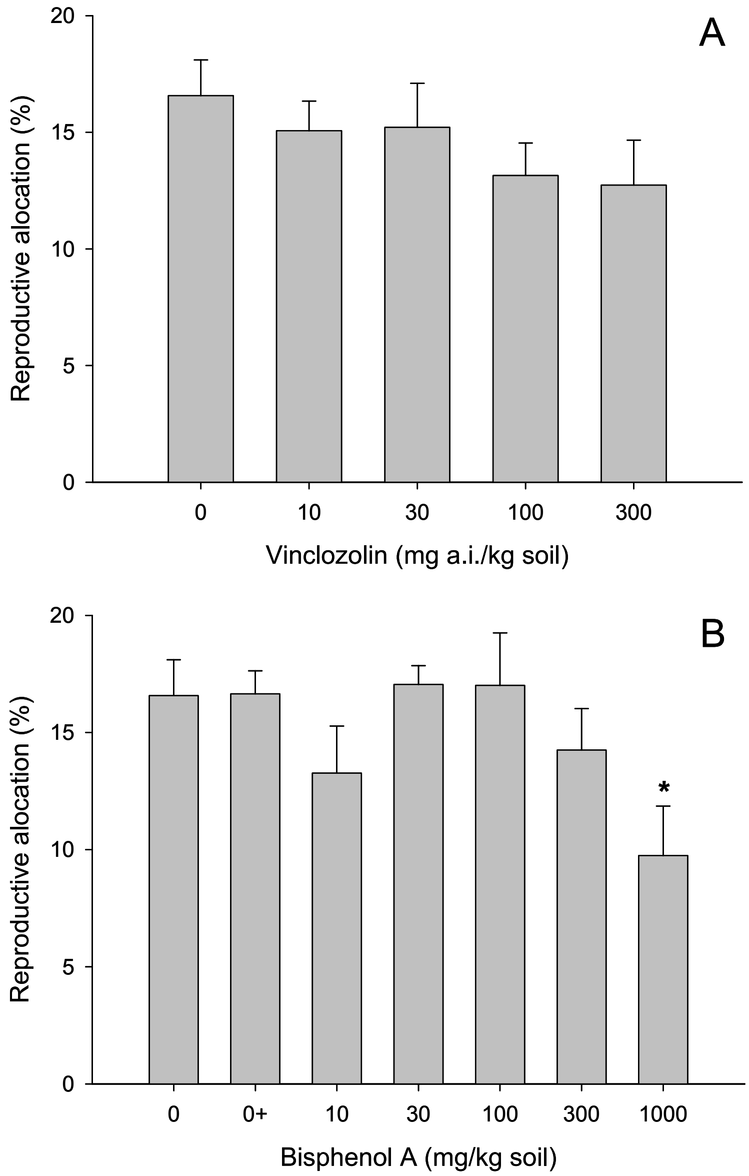

Figure 4.5 - Reproductive allocation of female *Porcellio scaber* exposed to soil treated with A) Vinclozolin and B) Bisphenol A. Vertical error bars represent the standard error of the mean. An asterisk indicates a significant difference from the control at $P \leq 0.05$ (ANOVA, Dunnett's test).

Chapter 5

Protein differential expression induced by endocrine disrupting compounds in a terrestrial isopod

5. PROTEIN DIFFERENTIAL EXPRESSION INDUCED BY ENDOCRINE DISRUPTING COMPOUNDS IN A TERRESTRIAL ISOPOD

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Abstract

Endocrine disrupting compounds (EDCs) have been studied due to their impact on human health and increasing awareness of their impact on wildlife species. Studies concerning the organ-specific molecular effects of EDC in invertebrates are still scarce and important to understand the mechanisms of action of this class of toxicants. This approach aims to unravel the protein expression in different organs of isopods exposed to Bisphenol A (BPA) and Vinclozolin (Vz) and assess their potential use as surrogate species. Male isopods were exposed to increasing concentrations of Vz and of BPA. After animal dissection, proteins were extracted from gut, hepatopancreas and testes. Protein profiles were analysed by electrophoresis and differentially expressed proteins were identified by MALDI mass spectrometry. EDCs affected proteins involved in the energy metabolism (arginine kinase), proteins of the heat shock protein family (Hsp70 and GRP78) and most likely microtubule dynamics (tubulin). Different proteins expressed at different concentrations in different organs' are indicative of the variety of possible higher-level of organization responses and the multi-level approach the ED problematic should have. Our findings suggest some common responses to EDCs in vertebrates and invertebrates, and points towards the potential use of these organisms' responses in ED risk assessment.

Keywords

Endocrine Disrupting Compounds • Proteomics • Soil invertebrates • Bisphenol A • Vinclozolin • Ecotoxicology

Briefs

Different organs from the isopod *Porcellio scaber* have differencial protein expression when exposed to the Endocrine Disrupting Compounds Bisphenol A and Vinclozolin.

5.1. Introduction

Bisphenol A (BPA) is a xenobiotic commonly employed in the manufacture of polycarbonate plastic and epoxy resins [1].
Although the plastic industry still claims that BPA is safe, its endocrine disrupting properties still raise concern [2]. In fact, BPA is known to act as a teratogen and may also lead to the alteration of sex determination and gonadal function [1]. In invertebrates, BPA induces various adverse effects, such as on the reproduction of the water fleas' *Ceriodaphnia dubia* and *Daphnia magna* [3, 4], and on the time to achieve sexual maturity of the copepod *Tigriopus japonicus* [5].
Vinclozolin (Vz) is a fungicide used mostly in turf grass and on vine plants. It is a proven endocrine disruptor which anti-androgenic effects are mainly due to its active metabolites, M1 and M2 [6]. In *D. magna*, Vz induced a decrease in the number of newborn males [7]. In molluscs, it induces the reduction of the number of ejaculated sperm cells, smaller testes, and disrupted male courtship behaviour [8]. Vz has also been reported to cause female virilisation (imposex development) and reduction of accessory sex organ expression in prosobranch snails [9].
Despite the increasing amount of information concerning the effects of endocrine disruptor compounds (EDCs) on vertebrates and on (marine) invertebrates, to our knowledge no data are available on the toxicity of BPA or Vz to edaphic organisms. Furthermore, for these compounds the mechanisms of effects at the molecular level are still largely unknown.
Woodlice are saprophytic detritivores, playing an important role in food webs, in the decomposition of organic material and in soil structuring [10]. Their biology and physiology is relatively well-known and they have been shown to be suitable test organisms for monitoring studies and to acquire individual toxicity data [11]. Their suitability for EDCs effect assessment have also been shown [12].
Most studies of endocrine disruption (ED) in invertebrates have been essentially focused in higher levels of biological organization. However, ED may in some cases arise secondarily as a result of pathological processes [13]. Therefore,

detailed insights into the mechanisms of toxicity are necessary to fully understand the mode of action (MoA) of these compounds.

In this work we developed a simple and accurate method to extract proteins from the organs of a terrestrial isopod. Also, we evaluated the protein expression of the gut, hepatopancreas and testes of organisms exposed to BPA and Vz.

5.2. Materials and Methods

5.2.1 Test organism and culture procedures

Isopods (*Porcellio scaber* Latreille, 1804) came from a culture established for more than 8 years. Animals in the culture were maintained as described in Lemos et al. [12].

5.2.2. Chemicals and preparation of soil

The natural soil, from an agricultural field kept in fallow from central Portugal, was oven dried at 60ºC for 48 hours and immediately weighed.

Bisphenol A (BPA, Merck Schuchardt, Germany, 2,2-bis-(4-hydroxyphenyl)-propane, purity >99 %) was dissolved in methanol and mixed with the soil at concentrations of 10, 30, 100, 300 and 1000 mg/kg dry soil. Afterwards, the soil was allowed to evaporate under a fume hood for 12 hours. Subsequently, soil moisture content was adjusted to 20% (v/w) with distilled water.

Vinclozolin (Ronilan® 50% active ingredient; BASF AG, Germany; 3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione) dissolved in water, was mixed in with the soil at concentrations of 10, 30, 100, 300 and 1000 mg a.i./kg dry soil. Soil moisture content was adjusted to 20% (v/w) with distilled water.

For control, moisture content was adjusted as described above. As for solvent control, the same volume of methanol without BPA was used, and soil was treated as described above.

Chemical analysis of Vz and BPA treatments concentration were made and were within ± 5% of the nominal concentrations. The results are presented in terms of the nominal values.

5.2.3 Organism exposure

Thirty male adult *P. scaber* per treatment were exposed to Vz and BPA for 14 days. Animals were randomly and individually placed in polyethylene terephthalate (PET) boxes (Ø100 mm x 50 mm) filled with 60 g of moist spiked soil and four Ø10 mm alder leaf discs.

5.2.4 Organism dissection

At the end of the experiments, five animals per concentration, undergoing intermolt, were dissected for gut, hepatopancreas and testes, on a frozen stainless steel plate in the presence of 100 mM Tris buffer pH 8.0 with PMSF (0.5 mM, Sigma). Each organ was immediately frozen in liquid nitrogen and kept at -80ºC until protein extraction.

5.2.5 Protein extraction and quantification

Each organ was suspended in buffer (8 M urea, 2% SDS, 100 mM Tris/Bicine) and homogenized. After centrifugation the supernatant was collected and kept at -20ºC. Samples were kept on ice throughout the extraction process. Protein concentration was determined using the BCA kit from Thermo Scientific, according to the manufacturer’s instructions.

5.2.6 SDS-PAGE

Proteins were denaturated and separated by SDS-PAGE according to Laemmli [14].The separation was performed in the Mini-PROTEAN 3 (Bio-Rad) with lab casted SDS polyacrylamide gels (15%). Gels ran for 2.5 h or 3 h, depending on the organ, at 125 volts. Proteins were visualized by Coomassie Brilliant Blue staining. Each gel image was acquired using the GS-710 calibrated imaging densitometer (Bio-Rad). Apparent molecular weights and band intensities were determined using the Quantity One v4.1 software (Bio-Rad). The apparent molecular weight of the proteins was estimated using a molecular weight calibration kit as marker, consisting of a mixture of proteins with 250, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa (Precision Plus Protein Standard, from Bio-Rad). In each gel, 3 lanes were loaded with the molecular mass standard and the molecular weights of the proteins were calculated using data from all standard lanes on each gel.

Band optical density was determined as (OD) / mm^2, subtracted for background and corrected for OD differences between gels.

5.2.7 Protein identification

5.2.7.1 Reduction and alkylation

Bands of interest were excised, reduced (10 mM dithiothreitol in 7 M GuHCl/0.3 M Tris, pH 9.0, 45 minutes at 55°C) and alkylated (55 mM iodoacetamide in 200 mM NH_4HCO_3 (pH 7), 45 min in the dark at room temperature) as described by Samyn et al. [15]. Removal of the excess iodoacetamide was accomplished by washing the gel pieces twice with 150 μl 50% acetonitrile/ultra-pure water (ACN/MQ). Finally, the gel plugs were dried in a Speedvac.

5.2.7.2 Trypsin digestion

To the dried gel plugs, buffer (50 mM ammonium bicarbonate, pH 7.8) containing modified trypsin/µL (Promega, Madison, WI) were added, and kept on ice for 45 min. Digestion was performed overnight at 37°C, the supernatant was recovered, and the resulting peptides were extracted twice with 60% ACN/0.1% N,N-diisopropyletylamine (DIEA). The extracts were pooled, dried in a SpeedVac and were redissolved in 0.1% TFA. One µL of sample was mixed with 1 µL matrix solution (7 mg/ml α-cyano-4-hydroxycinnamic acid solution in 0.1% TFA/50% acetonitrile) and mixture was spotted on a MALDI-plate. The plates were allowed to air-dry at room temperature, and were then inserted in the mass spectrometer and subjected to mass spectrometric analysis.

5.2.7.3 Matrix-Assisted Laser Desorption/Ionization TOF/ TOF Mass Spectrometry

The Applied Biosystems 4800 Proteomics Analyzer with TOF/TOF optics was used in this study for reflectron analysis and MALDI MS/MS applications (Applied Biosystems, Foster City, CA, USA). The mass spectrometer uses a 200-Hz frequency tripled Nd:YAG laser operating at a wavelength of 355 nm.

Prior to analysis, the mass spectrometer was externally calibrated with a mixture of Angiotensin I, Glu-fibrino-peptide B, adrenocorticotropic hormone (ACTH) (1-17), and ACTH (18-39). For MS/MS experiments, the instrument was externally calibrated with fragments of Glu-fibrino-peptide. MS and MS/MS data were further processed using DataExplorer 4.0 (Applied Biosystems) or by manual interpretation.

5.2.8 Statistical analysis

Results are presented as mean ± SE. All data were checked for normality and homoscedascity. One way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means were employed to determine significant differences relatively to the control treatment. For all statistical tests the significance level was set at $P<0.05$. Calculations were performed with SigmaStat (Systat Software Inc, California, USA).

5.3. Results

Protein extraction of the hepatopancreas, testes and gut.

Although there is no lethal effect of BPA and Vz after 15 days of exposure at these concentrations (LC_{50} is > 1000 mg/kg of soil), exposure to the chemical elicited a protein response in all organs.

The extraction method was adequate as protein profiles were seen to be similar within each organ/toxicant concentration. After image analysis of at least 5 replicates per organ per toxicant concentration, several bands, with clearly different intensity compared to controls were selected (figures 5.1-5.3). The intensities of these proteins were analysed in all gels and average intensities are shown in figures 5.1a- 5.3a (for Vz treated organisms) and in figures 5.1b-5.3b (for BPA treated organisms).

Exposure to bisphenol A

In the hepatopancreas, two proteins were significantly over-expressed by the highest and lowest concentration tested (10 and 1000 mg/kg of soil; figure 5.1b) (ANOVA, Dunnett's test, $F_{5.25} = 3.703$, $P = 0.012$ and $F_{5.24} = 5.024$, $P = 0.003$ for 68.5 and 38.8 kDa respectively).

The gut protein profiles showed that from the bands detected, one (74.9 kDa) was up-regulated with increasing concentrations of BPA (figure 5.2b) with a LOEC of 300 mg/kg of soil (ANOVA, Dunnett's test, $F_{5.20} = 5.049$, $P = 0.009$).

Considering the testes of BPA exposed organisms, three proteins (molecular weights of 75.8, 73.0 and 53.8 kDa) were significantly over-expressed (figure 5.3b). Heavier proteins (75.8 and 73.0 kDa) were up-regulated at concentrations of 30 and 100 mg/kg of soil (ANOVA, Dunnett's test, $F_{5.20} = 4.057$, $P = 0.011$ and $F_{5.19} = 5.483$, $P = 0.006$ respectively). The 53.8 kDa protein was over-expressed with increasing concentrations with a NOEC of 100 mg/kg of soil (ANOVA, Dunnett's test, $F_{5.19}$= 4.646, P= 0.006).

Exposure to vinclozolin

Differences in the testes, hepatopancreas and isopods' gut protein expression were observed. Both testes and hepatopancreas showed three proteins up-regulated (figures 5.1a and 5.2a). Nevertheless, while in the hepatopancreas up-regulation had a LOEC of 1000 mg a.i./kg of soil for all three proteins, in the testes up-regulation occurred and was significantly different at the lowest concentrations tested, 10 and 30 mg a.i./kg of soil for the 75.8 kDa protein (ANOVA, Dunnett's test, $F_{3.12}$= 5.285, P= 0.015) and 10 mg a.i./kg of soil for the 73.0 and 62.6 kDa proteins (ANOVA, Dunnett's test, $F_{3.13}$= 8.3, P= 0.002 and $F_{3.14}$= 12.496, P< 0.001 respectively) (figure 5.1a). In the gut one protein with a molecular weight of 74.9 kDa was over-expressed, being statistically significant comparing to control at the concentration of 1000mg Vz/kg of soil (figure 5.3a) (ANOVA, Dunnett's test, $F_{5.19}$= 5.918, P= 0.002).

Protein identification

The proteins which showed statistically different expressions among treatments were selected for identification. Each band was manually excised from the gel, trypsin digested and analysed by MALDI-TOF MS. Only the PMFs that were confirmed by MS/MS were considered as a positive identification. An obvious limitation of working with non-model organisms is that since their genome is not known, proteins sequences are absent from the databases and only some proteins can be positively identified. Therefore, whenever necessary, *de novo* sequencing of proteins was performed. Nevertheless, from the 12 potential proteins that were significantly over-expressed we were able to make a positive identification of 5 proteins (table 5.1).

5.4. Discussion

Endocrine, immune and neuronal systems as well as reproductive organs are frequently pointed out as targets of EDCs in both vertebrates and invertebrates [16]. Nevertheless, EDCs MoA and concentrations at which organisms' responses are observed, have not yet been completely clarified and remain a fundamental issue for toxicologists [17].

Protein profile changes in response to specific chemicals can provide useful marker proteins against specific chemicals, and have been detected in organisms submitted to chemical stressors [16]. This technique has primarily benefited the research on well-characterised species such as human, mouse and yeast. Apart from clinical data, reports focusing on protein profile alteration in response to toxicants are still quite scarce [18]. The use of proteomics for studies of ecologically relevant species is not impossible, but in the cases where no genome sequencing data is available, it entails more work and more costs for less information. The lack of sequenced genomes hampers the construction of DNA microarrays and increases the complexity of protein identification by MS technologies. In fact, contrasting with the early start of studies in non-model organisms exposed to selected pollutants, and the ensuing studies in model organisms, very few proteomic studies exist yet in animals from natural environments [19]. But, as Hutchinson [20] stated, "the practical science [proteomics] is still in its infancy" and studies like the present one should broaden our knowledge on the effects of toxicants at the protein level of these organisms.

In its origins, the term "proteomics" was used to describe two-dimensional gel electrophoresis (2-DE) and mass spectrometric analyses of proteins of interest, but nowadays the term has been applied to any parallel method of protein analysis [21]. In this study we have begun a search for proteins that are differentially expressed in particular organs of *P. scaber* after exposure to Vz and BPA. The terrestrial isopod gut is where digestive processes mainly take place, whereas the hepatopancreas (digestive gland) secretes digestive fluids into the hindgut and is involved in the absorption of digestively released nutrients and is the main factory of proteins with a wide broad of action [22], having intestinal, hepatic, and pancreatic functions [23]. Significant changes in protein profiles of each of these organs may lead to effects at the individual level but also at the population level. This is especially relevant for gonads. If chemically exposed isopods show gonad alterations, it may affect breeding and most probably affect isopods' population dynamics.

This study revealed significant differences in all tested organs of *P. scaber* after BPA and Vz exposure. However, the sensitivity of each organ was not the same. In *P. scaber* testes it was possible to detected up-regulated proteins at Vz concentrations as low as 10 mg a.i./kg soil while for hepatopancreas this was only possible at concentrations equal to 1000 mg a.i./kg soil. A similar pattern of gonad sensitivity was found for BPA exposed organisms, while for the gut and hepatopancreas altered protein profiles were only detected at BPA concentrations higher than 300 and 1000 mg/kg, respectively. Testes showed increased protein expression at concentrations higher than 30 mg/kg soil. This suggests that testes are more susceptible to these compounds than other organs, showing that the male isopod reproductive traits may therefore be especially susceptible and sensitive to this class of chemicals. Previous studies have also stressed the gonads and reproductive traits as preferential targets by EDCs in vertebrates [24]. An important aspect concerning the ecological relevance of EDC studies on invertebrates is the possibility of establishing useful biomarkers [25]. The low concentrations at which protein profile alterations were detected in the testes (figures 4a and b) not only are far from those which are lethal to *P. scaber*, but also were observed earlier than reproductive, developmental and molting effects were (Lemos et al, unpublished data). On the other hand, effects at low concentrations, as seen in our investigation, are in agreement with the literature that has shown that some of these EDCs produce effects with humped dose-response curves, with tests conducted at high doses missing biological effects that are induced by lower doses [26].

Our data shows an up-regulation of Hsp70 proteins (known as ubiquitous stress response proteins) in the testes of organisms exposed both to Vz (around 160% increase after exposure to 10 mg a.i./kg and around 130% at 30 mg a.i./kg) and BPA (around 120% increase at 30 and 100 mg/kg). The induction of Hsp70 proteins has been reported in a wide range of organisms, from microorganisms to humans, upon exposures to various kinds of chemical, physical and biological stressors [27]. In this study, they were over expressed by the testis at low concentrations, therefore representing a typical molecular response to EDC exposure. Hsp70 proteins are anti-apoptotic proteins, protecting cells from cytotoxicity and inhibiting cell death induced by several agents [28]. Increases in Hsp70 protein concentration aid refolding damaged proteins during stress by a process called 'unfolded protein response' [29]. Hsp70 stabilizes unfolded protein precursors before folding and assembly occurs, and it is involved in the translocation of unfolded protein.

Additionally, the fact that Hsp70 was induced by both toxicants suggests that it could be used as a biomarker of exposure to low concentrations of chemicals in the testes. This response is not stressor specific but should be further investigated and validated as a general biomarker of environmental quality. Such a suggestion has also been made for *Mytilus* sp., where a significant up-regulation of Hsp70 after exposition to BPA was observed [30]. BPA also induced an increase of Hsp70 mRNA in the aquatic larvae of *Chironomus riparius* [31].

In testes exposed to BPA, the Hsp70 protein was identified as glucose-regulated protein 78 (GRP78). This family of molecular chaperones is located in the lumen of the endoplasmic reticulum (ER) and is induced by stress [32]. An up-regulation of this protein seems to be related to the accumulation of unfolded proteins in the ER [29] and protects cells against toxic insults [32]. This stage of induction of these ER stress proteins corresponds to a mild ER stress, which is followed by a series of cellular events and If the disruption of ER function is not restored to homeostasis, apoptosis is initiated through the activation of cell death pathways [33].

It has also been shown that GRP78 plays an important role in the survival of cells during calcium stress, being induced by depletion of stored calcium. An elevated intracellular calcium concentration induced by BPA in mouse hippocampal neuronal cells was shown previously [34]. Also, in Sertoli TTE3 cells, BPA mobilizes intracellular calcium inducing ER stress [35]. Furthermore, GRP78 has been seen to be induced by natural estrogens in the mouse uterus via the oestrogen receptor (ER)-independent mechanism [36] and that it is involved in the control of ER gene expression, able to amplify estrogenic potency of weak xenoestrogens.

This up-regulation of GRP78 in isopod testes is an indication that in invertebrates similar molecular events might occur as in vertebrate cells exposed to BPA.

For BPA exposed isopod testes, there was an increase of β-tubulin with increasing concentrations (NOEC 100 mg/kg dry soil). β-tubulin and α-tubulin are the components of microtubules in eukaryotic cells [37] playing a central role in many aspects of cell function, including cell motility and division [38]. During cell division (mitosis or meiosis) microtubule dynamics play an essential role in the proper orientation and segregation of chromosomes. Impairment in the functioning of microtubules leads to an abnormal morphology of the cells and may lead to apoptosis [39]. An interaction between tubulin and BPA in eggs of the urchin (*Lytechinus pictus*) was reported earlier [40]. These authors have identified tubulin as a direct target of BPA, affecting microtubule assembly, suggesting that BPA induces the *de novo* formation of ectopic asters. These results are in agreement with our data that show an over-expression of tubulin in the testes. Such up-regulation may cause mitotic or meiotic aneuploidy [40]. Exposure of mouse oocytes to BPA affected spindle formation, distribution of pericentriolar material and chromosome alignment on the spindle, and caused a significant meiotic arrest [41]. In isopod testes this could have implications for gametogenesis and therefore have a major impact on isopod reproduction and most certainly lead to effects at the population level.

Arginine kinase (AK) is widespread in invertebrates, where it serves a function analogous to that of creatine kinase in vertebrates [42] and is considered to be the primordial enzyme among phosphagen kinases [43]. This enzyme is involved in the cellular energy metabolism, catalysing the reversible formation of arginine phosphate and adenosine diphosphate from ATP and L-arginine [42] and therefore responsible for ATP buffering in the cytosol and energy shuttle between mitochondria and the cytosol [44]. This buffering function of the phosphagen kinases seems to be characteristic in tissues with short bursts of energy demand

[45]. It has been demonstrated that arginine phosphate can act along with AK as a temporal energy buffer system in *Locusta migratoria* [46]. After Vz and BPA exposure we detected an up-regulation of AK in isopod hepatopancreas (around 150% at 1000 mg a.i./kg for Vz and approximately 120 and 130% in isopods exposed to 10 and 1000 mg BPA/kg). The hepatopancreas not only is the major digestive organ of isopods, as it also is the main site for synthesis and secretion of digestive enzymes, absorption of nutrients, storage of metabolic reserves and excretion of wastes [23]. The up-regulation of a molecule involved in the energy metabolism of the organism might be due to the stress response to toxicant exposure, with the activation of metabolic processes related to detoxification and metabolisation of energy reserves to provide for those energy-demanding processes [47]. In fact, *P. scaber* exposed to high concentrations of Vz and BPA grew less (Lemos et al., unpublished data) and had lower reproductive effort (Lemos et al., unpublished data) compared to control organisms. This reduced growth might be caused by the suggested ED mechanisms but also as a general toxicity response with fitness reduction or by depletion of energy reserves to sustain metabolic processes related to detoxification. Because AK plays an important role in energy metabolism, its up-regulation seems to indicate that there is a substantial energetic cost in terms of hepatopancreas ATP demand after Vz and BPA exposure.

The understanding of mechanisms that take place in cells due to toxic effects of EDCs is far from being complete. The approach developed in the present study provides evidence that Vz and BPA can alter the expression of different protein profiles in different organs of a soil invertebrate. To our knowledge, this is the first demonstration of such effects on the isolated organs of terrestrial arthropods. In this work, the use of MS/MS and *de novo* sequencing enabled the identification of several proteins that were over-expressed. Although the traditional enzyme and other molecule biomarker approaches might reveal a higher sensitivity to specific targets, this approach enables the assessment of a vaster array of molecular targets, which will provide researchers with the full scope of the effects of the chemicals even when no prior knowledge of the chemicals' mode of action is available.

Certainly, other protein expression changes occurred in our experiments, but they were either too small to be detected or concerned less abundant proteins. To obviate such an issue, fluorescent 2D differential in gel electrophoresis (DIGE) should be addressed.

Both compounds tested affected the energy metabolism and induced a variety of general stress responses in the isopod. The interaction of EDCs with tubulin in the gonads may lead to aneuploidies and consequently to the impairment of the overall reproductive function and therefore may affect the population dynamics.

Chemical signalling systems and molecular mechanisms in the animal kingdom are known to exhibit a considerable degree of conservatism (McLachlan, 2001). Consequently, invertebrates can be presumed to be subject to modulation by identical or similar compounds as in vertebrates (Pinder and Pottinger, 1998). Our findings are in agreement with this by suggesting a common response of some proteins by vertebrates and invertebrates. Such similar responses hint towards the use of isopods as potential surrogate species for both invertebrates and even vertebrates in environmental risk assessment.

It should be stressed that this methodology enabled identification of alterations of the expression of endocrine related proteins (such as GRP78), as well as ED typical non-monotonic dose responses. Similar responses of the individuals' exposed to different concentration of a toxicant may have different causes at a lower level of biological organization. Thus, carefully focused research programs using proteomics, may prove it to be a powerful tool towards unravelling the molecular mechanisms that underlie these "odd low-dose responses".

Further studies are needed and we are still far away from attaining full knowledge on how endocrine disruptors exert their action in living organisms. Research focusing on the cellular effects of these compounds in terrestrial organisms is essential and will increase our knowledge of how endocrine disruptors may affect wildlife and ecosystems.

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Tables

Table 5.1. - Identification of differentially expressed proteins by mass spectrometry in the isopod *Porcellio scaber* after 15 days of exposure to Vinclozolin or Bisphenol A in soil. The apparent molecular weight of proteins was determined by SDS-PAGE. Protein identification was achieved by mass spectrometry.

Toxicant	Organ	Apparent MW (kDa)	Protein	Analysis method
Vinclozolin	Hepatopancreas	38.2	Arg kinase	PMF, MS/MS and *de novo*
		16.3	*n.i.*	-
		14.6	*n.i.*	-
	Testes	75.8	*n.i.*	-
		73.0	Hsp70	PMF, MS/MS
		62.6	*n.i.*	
	Gut	74.9	*n.i.*	-
BPA	Hepatopancreas	68.5	*n.i.*	-
		38.6	Arg kinase	PMF, MS/MS and *de novo*
	Testes	75.8	GRP78	PMF, MS/MS and *de novo*
		73.0	*n.i.*	-
		53.8	Beta-tubulin	PMF, MS/MS and *de novo*
	Gut	74.9	*n.i.*	

n.i.: protein not identified

Figures

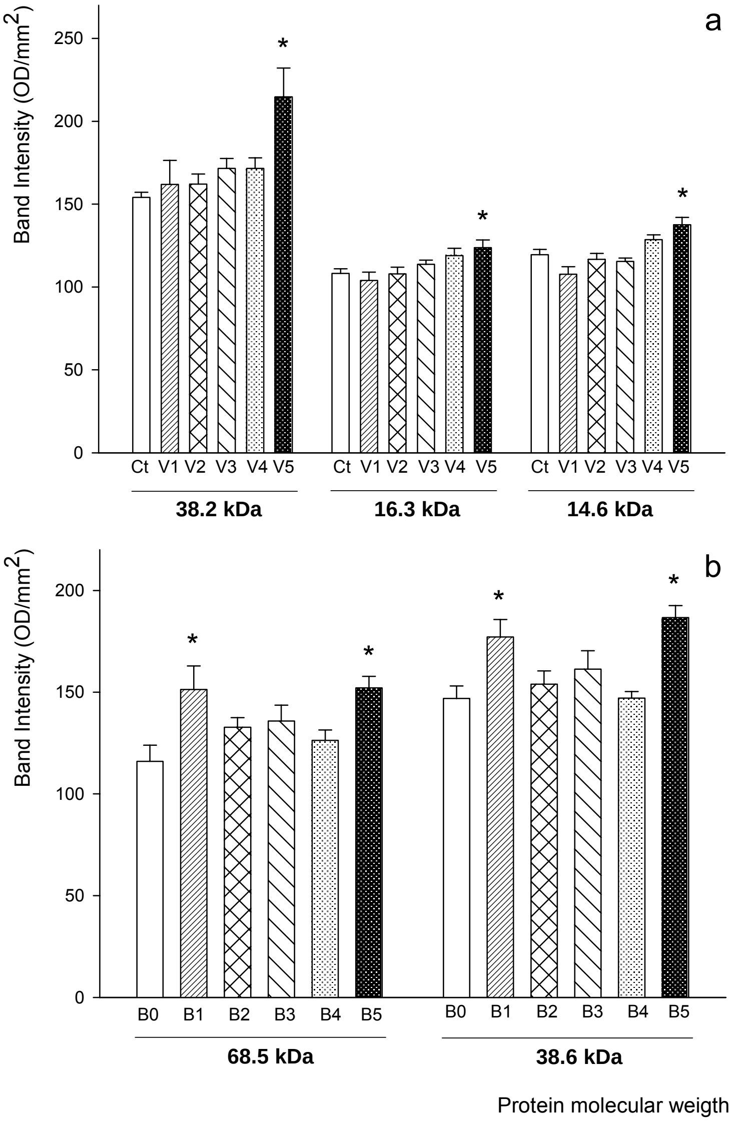
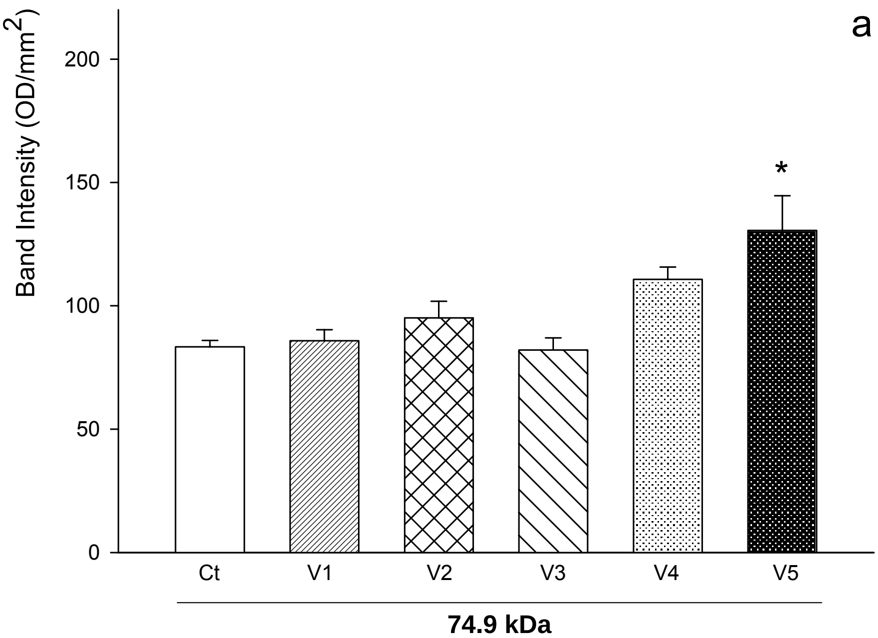


Figure 5.1 - Effect of toxicant exposure on protein expression in the hepatopancreas of *Porcellio scaber*. a - Expression of proteins with molecular weights of 75.8, 73.0 and 53.4 kDa of the hepatopancreas of isopods exposed to Vz contaminated soil, expressed as intensity of bands. b - Expression of proteins with molecular weights of 68.5 and 38.6 kDa of the hepatopancreas of isopods exposed to BPA contaminated soil, expressed as intensity of bands. An asterisk indicates a significant difference from the control at P ≤ 0.05 (ANOVA, Dunnett's test).



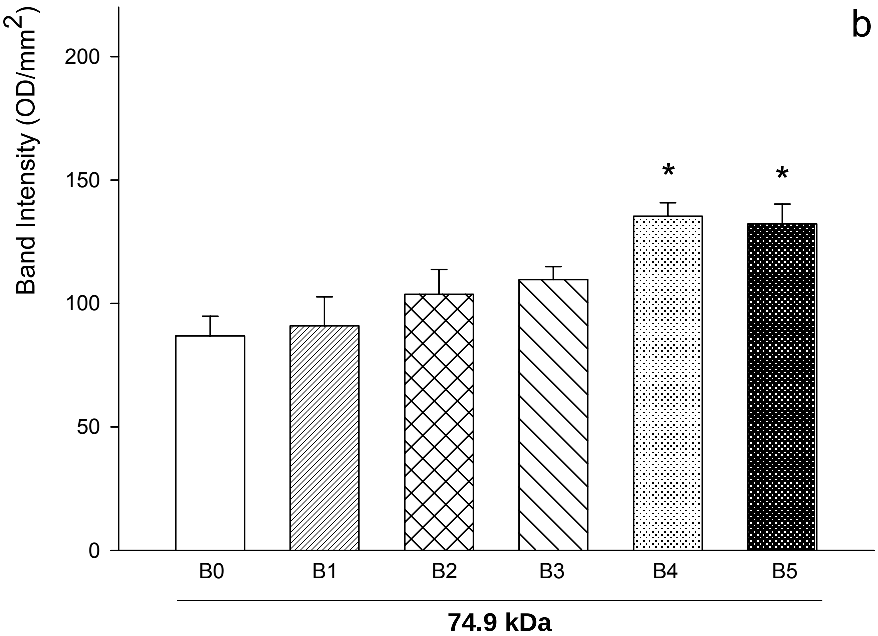


Figure 5.2 - Effect of toxicant exposure on protein expression in the gut of *Porcellio scaber*. a - Expression of proteins with molecular weights of 74.9 kDa of the hepatopancreas of isopods exposed to Vz contaminated soil, expressed as intensity of bands. b - Expression of the protein with molecular weight of 74.9 kDa of the gut of isopods exposed to BPA contaminated soil, expressed as intensity of bands. An asterisk indicates a significant difference from the control at $P \leq 0.05$ (ANOVA, Dunnett's test).

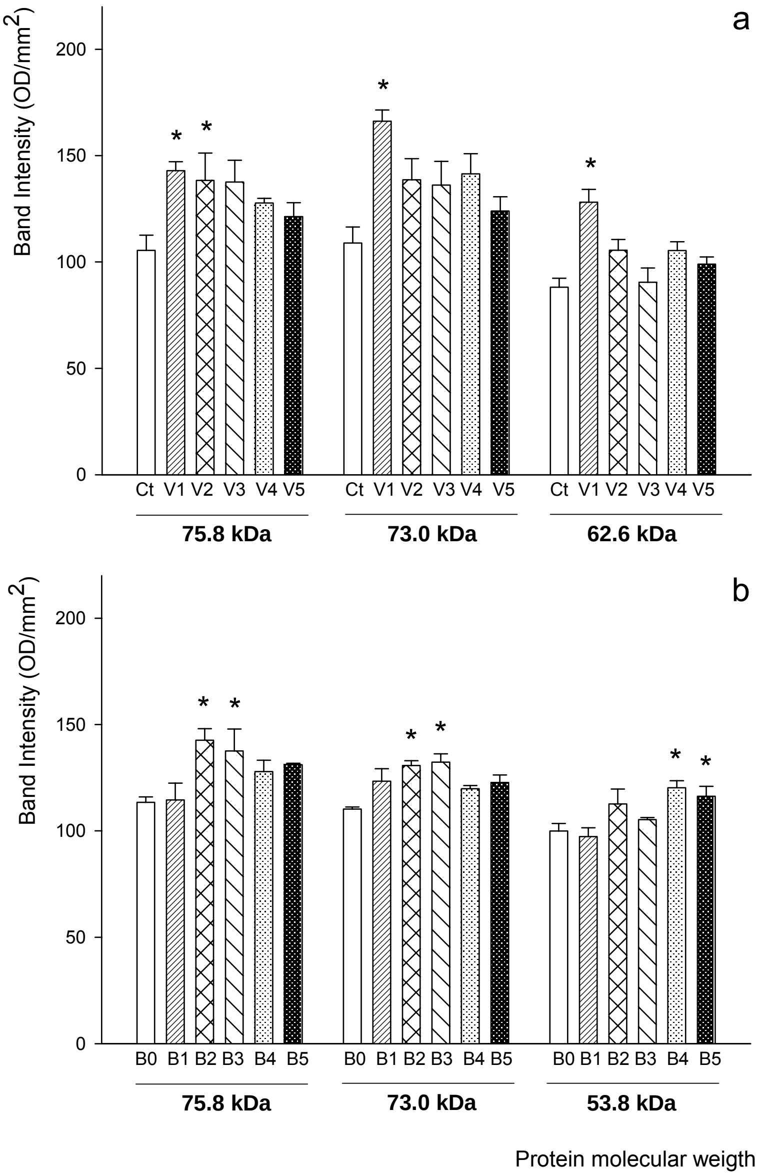


Figure 5.3 - Effect of toxicant exposure on protein expression in the testes of *Porcellio scaber*. a - Expression of proteins 38.2, 16.3 and 14.6 kDa of the testes of isopods exposed to Vz contaminated soil, expressed as intensity of bands. b - Expression of proteins with molecular weights of 75.8, 73.0 and 62.6 kDa of the testes of isopods exposed to BPA contaminated soil, expressed as intensity of bands. An asterisk indicates a significant difference from the control at $P \leq 0.005$ (ANOVA, Dunnett's test).

Chapter 6

General conclusions, considerations and research perspectives

6. General conclusions, considerations and research perspectives

The focus of this research was to investigate the effects of exposure to Vinclozolin and Bisphenol A, an anti-androgen and a xeno-oestrogen, respectively in major physiological processes of a terrestrial invertebrate. Potential mechanisms that underlie the reproductive and developmental toxicity were investigated. An evident causal link with ED was addressed as well as other mechanisms of ecotoxicity.

Since chemical signalling systems and their basic mechanisms in the animal kingdom exhibit a considerable degree of conservatism, it could be presumed that endocrine systems in invertebrates would be subject to modulation by identical or similar compounds as in vertebrates.

Crustaceans represent the systematic group within invertebrates that provide the majority of ED case studies. Nevertheless, while the examples for the aquatic environment are almost balanced between freshwater and marine species, to our knowledge, until now there were no reports of comparable studies on terrestrial crustaceans.

Vz and BPA were first documented as endocrine disruptors in vertebrates. While they have already been evaluated in some aquatic invertebrates, no information existed for the soil compartment.

Isopods like *Porcellio scaber* combine the features of continuous growth, through a molting regime, and reproductive processes mediated by well known endocrine mechanisms with a terrestrial mode of life, making them strong candidates for sentinel species for these types of pollutants in terrestrial environments.

Therefore, in order to study the effects of exposure to Vz and BPA, the first step was to evaluate the ecdysteroidal disrupting capability of the two model EDCs and in this way prove that they are true EDCs to *P. scaber*.

Ecdysteroids are mostly known for their role in regulating molting but they are also chief hormonal factors controlling reproduction in isopods. 20-hydroxyecdysone concentration was assessed after 7, 14 and 28 days exposure to soil contaminated with Vz and BPA. Results demonstrated that both chemicals exhibited ecdysteroidal activity in *P. scaber* by increasing ecdysone endogenous levels in a concentration-dependent way. An exception was found for BPA at the lowest concentration (10 mg/kg dry soil) for which high levels of 20E were also found, suggesting the presence of "low dose" effects typical of endocrine disruptors.

Sex-ratio disorders were also found for BPA. The lowest concentration of BPA (10 mg/kg dry soil) significantly skewed the gender ratio to one male for every two females, contrary to a near one male per every female in non-exposed isopods. Nevertheless, the parsimony principle withstands the simpler and straightforward explanation that the skewed ratio could simply be due to differential lethality of genders to the toxicant with a higher mortality among males. The issues raised could be tackled in the future by performing gonad histopathology in exposed isopods, assessing abnormalities associated with masculinised females or feminized males, or by identifying genetic sex markers that can be used to compare genetic sex with individual phenotypic secondary sexual characters.

Since a sharp rise of ecdysteroid followed by a decrease of the hormone level triggers the ecdysis process, when basal concentration of 20E is maintained at high levels (hyperecdysonism) the shedding of the old cuticle is impaired and mortality due to incomplete ecdysis occurs. Therefore, molting behaviour was associated with hyperecdysonism, and although both chemicals induced changes in molting parameters, while BPA precipitated molt, highest concentrations of Vz postponed molt, which is intimately associated with the lack of success to complete molt, ending up in death of the organisms.

Life-stage dependent responses were also assessed. Juveniles followed the same trend for anticipating molt when exposed to BPA and to postpone it when exposed to Vz contaminated soil. Furthermore, juveniles showed to be more sensitive to the toxicants. This was revealed not as much by the LCs for effects on survival but rather for the LOECs for effects on growth. Nevertheless, in the long-term, for both juveniles and adults, overall growth was impaired by both chemicals.

Having in mind that the ultimate goal of ecotoxicologists is to monitor and/or predict the effects of toxicants on the long-term health of individual organisms, populations, communities and ecosystems, the need of juvenile testing due to their higher sensitivity has been demonstrated here and is therefore highly recommended.

In this work, it was not possible to distinguish if the increased sensitivity of females over male isopods, found in BPA exposed juveniles, was due to endocrine gender specific differences or due to the different trade-offs based on the allocation of resources available for future reproduction. For this reason the research presented here emphasised the importance of assessing toxicant effects on males and females separately.

The two selected EDCs elicited an overall reproductive toxicity in terrestrial isopods with decreasing reproductive allocation for the exposed females. Vz reduced pregnancy duration, increased the abortion percentage, decreased the number of pregnancies, and decreased of number of juveniles per female while BPA increased abortions at the lowest and highest exposure concentrations (10 and 1000 mg/kg dry soil).

The high levels of abortions and unsuccessful pregnancies were in accordance with the 20E level variation, which was indicated as a possible cause of embryo development toxicity. Ecdysteroids, as chief hormonal factors, are known to be necessary for vitellogenin synthesis. The coordinated control of molting and reproduction is achieved by this ecdysteroid/vitellogenin inter-relationship mechanism. Thus, the 20E variation found must most probably interfere with vitellogenic processes, which may be underlying the reproductive impairment detected. Moreover, in crustacean females, the reproductive cycle is inextricably linked with the molt cycle. For this reason, the integration of molting with reproduction is a physiological need in female crustaceans.

The stimulatory effects of 20E on vitellogenin and the importance of ecdysteroids on the molt cycle are two of the main reasons for the overall reproductive impairment and explain the causal link with ED. Nevertheless, it was also considered possible that since parental care by female isopods is an energetically demanding process, resources might have been deviated from reproduction to insure increased costs of basal metabolism.

Effects of the two EDCs on reproduction and development were determined and its causal link to endocrine disruption was proven. Nevertheless, it is certain that these compounds, especially at higher concentrations, are due to cause other effects. For this reason, we aimed at using a non-specifically targeted methodology to assess the ecotoxicological mode of action of BPA and Vz. Although the traditional enzyme and other molecule biomarker approaches might reveal a higher sensitivity to specific targets, the proteomic approach developed in this study enabled the assessment of a broader array of molecular targets with no prior knowledge of the chemicals' mode of action.

This methodology allowed to show that both compounds tested affected the energy metabolism and induced a variety of stress responses in the isopod. Arginine kinase is involved in the cellular energy metabolism and both Vz and BPA caused its over-expression in the isopods' hepatopancreas. This suggests an increase of resources allocated to the activation of metabolic processes related to detoxification and the metabolisation of energy reserves to provide for these processes.

The male gonad of *P. scaber* seems to be the most sensitive organ tested. Proteins from the heat shock protein family, Hsp70 and GRP78, were over-expressed at the lower concentration of BPA and Vz respectively. Moreover, BPA caused an up-regulation of tubulin in the testes. Interaction between BPA and tubulin has been related to aneuploidies. This tubulin up-regulation combined with the organs' higher sensitivity should inspire concern since it may lead to the impairment of reproductive function and may therefore affect the population dynamics of the test species.

More protein expression alterations are certain to have occurred in our experiments, but they were either too small to be detected or concerned less abundant proteins. In order to obviate such an issue, other more sophisticated methodologies, such as fluorescent 2D differential in gel electrophoresis (DIGE), should be tested in the future.

Nevertheless, the present fairly simple approach allowed the detection of several proteins that were differentially expressed due to toxicant exposure.

These findings suggest a common response to BPA by vertebrates and invertebrates. Such similar responses hint towards the use of isopods as potential surrogate species for both invertebrates and even vertebrates in environmental risk assessment.

While the overall results are indicative of the chronic toxicity of these compounds, the exposure concentrations at which these effects were elicited are well above the ones expected to be found in the environment and at a first glance pose no threat to natural populations of these terrestrial organisms. Nevertheless, natural and synthetic hormone mimics are widespread in the environment and when having an identical mode of action (e.g. acting through the same receptor) risk assessment might become problematic. Therefore, further research is needed to determine whether environmental EDCs, whose effects are suspected to be additive or synergistic, are present either alone or in combination at environmental concentrations high enough to impact natural populations.

This is the first study reporting “ED typical non-monotonic dose-responses” in edaphic invertebrates, which ought to have special attention from ecotoxicologists because they challenge a fundamental pillar of toxicology, the classic dose-response curve. Similar responses of individuals exposed to different concentrations of a toxicant may have different molecular causes. Thus, carefully focused research programs using molecular tools such as proteomics may prove to be helpful in unravelling the molecular mechanisms that underlie these odd low-dose responses. Furthermore, research focusing on the molecular effects of endocrine disrupters in terrestrial organisms is essential and will increase knowledge of how these compounds exert their action in living organisms and impact wildlife and ecosystems.

Effects at the population/community level are only detected in most cases after several generations exposed to sub-lethal levels of pollutants and effects studied in conventional ecotoxicity tests (changes in growth, reproduction and survival) can be considered as a final result of the cumulative molecular and cellular effects. Therefore, there is an urgent need to develop and validate quantifiable tools of fast detection (“early warning signals”), which can foresee any changes in the structure of the population/community, and that work through the inhibition of vital processes in particular species. These biomarkers might be considered as a measure of the first toxicological interactions between the toxicant and the biological receptor. This interaction induces a cascade of events, starting at sub-cellular level (e.g., gene transcription disturbances, metabolic path interference, etc.) that, in last instance, lead to hazardous effects at the individual level that may ultimately lead to population decrease and extinctions. Recovery from such impacts might be difficult, even after the regulation of activities involving these compounds.

In parallel to this aspect of evaluating the effects of EDCs and to develop and validate these early warning tools for non-target soil organisms, there is also the need to transfer this knowledge to more realistic exposure scenarios. This is particularly true in cases where multiple exposures can occur, especially when assessing the toxic potential of contaminated sites. In this context, *in situ* tests have a promising potential. The data gathered on these types of bioassays does not require prior knowledge of the type or the concentrations of chemicals present in the soil. Moreover it allows the assessment of the toxicity of complex mixtures and integrates a complexity of factors, such as effects of exposure time and exposure conditions. As such, they have the advantages of laboratory assays without the need for extrapolation to effects in the field.